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13. ABSTRACT (Maximum 200 Words)

This study was aimed at evaluating the enhanced antitumor response to radiotherapy by poly(L-glutamic acid)-paclitaxel (PG-TXL) conjugate. We compared the ability of paclitaxel and PG-TXL to sensitize radioresponse using both tumor growth delay and tumor curability as the end points. Furthermore, the effect of treatment schedule on the radiosensitizing activity of PG-TXL as well as the effect of PG-TXL on the sensitivity of normal tissues to radiation was also studied. Our data demonstrate significant synergistic interaction between PG-TXL and tumor radiation. Compared to paclitaxel, PG-TXL enhanced the response of tumors to radiation for approximately 4 folds. Importantly, the enhanced antitumor activity was achieved without apparent effect on the normal tissues. Our results also suggest that PG-TXL may exert its radiosensitizing effect through multiple mechanisms, one of which being sustained release of paclitaxel in the tumor from PG-TXL. This study has lead to a National Cancer Institute-sponsored clinical study that has been initiated at the University of Texas M. D. Anderson Cancer Center.

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Radiation Induced Chemosensitization: Potentiation of Antitumor Activity of Polymer-Drug Conjugates

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INTRODUCTION

The combination of chemotherapy and radiotherapy has become a common practice in the therapy of locally advanced cancer. However, chemoradiotherapy is often associated with increased toxicities to normal tissues that limit its application. One approach to improve the therapeutic index is to increase the selective delivery of chemotherapeutic agents to the tumors. Towards this goal, we have developed a polymeric drug, poly(L-glutamic acid)-paclitaxel (PG-TXL), that allows higher concentrations of paclitaxel (TXL) to be delivered selectively to tumors (Reviewed in Reference 1). Recent studies show that taxanes are potent enhancers of *in vitro* tumor cell radiosensitivity and *in vivo* tumor radioresponse (Reviewed in Reference 2). The degree of enhancement with both docetaxel and TXL are limited to about 2 folds (2). To further improve the radiosensitization effect of taxanes, we hypothesize that

I: Radiation may potentiate tumor response to PG-TXL by increasing tumor vascular permeability towards macromolecules and thus increasing tumor uptake and therapeutic efficacy of PG-TXL; and

II. A sustained release drug delivery system that localize the drug to the tumor and release it slowly over time, such as intravenously injected PG-TXL, would enhance the responsiveness of solid tumors to radiotherapy.

Our results demonstrate that combined PG-TXL and tumor irradiation produced remarkable synergistic antitumor effect. Moreover, such an effect was achieved without increased toxicity to normal tissues. As a result of our study, a Phase I/II clinical trial of PG-TXL in combination with radiotherapy for the treatment of non-small lung cancer has been initiated at M.D. Anderson Cancer Center.

BODY

We propose a treatment strategy combining radiation and macromolecular chemotherapy that may have important clinical implications in terms of scheduling and optimization of the therapeutic ratio. To test our hypotheses, we first studied the enhanced antitumor efficacy of combined therapy using tumor growth delay as the end point and a treatment schedule in which tumor irradiation preceded the administration of PG-TXL (3). Our data demonstrated that local tumor irradiation enhanced the distribution of PG-TXL given 24

h later to ovarian OCa-1 carcinoma implanted intramuscularly in C3Hf/Kam mice. Radiation significantly increased tumor uptake of PG-TXL and tumor vascular permeability, caused elevation of the serum concentration of vascular endothelial growth factor (VEGF), and arrested OCa-1 cells in G1 phase of cell cycle. The enhancement factors as measured by incremental tumor growth delay compared with PG-TXL alone ranged from 1.36 to 4.44. Complete tumor regression was also observed at higher radiation dose (>10 Gy) and higher PG-TXL dose (>80 mg eq. TXL/kg). Furthermore, combined radiation and PG-TXL produced significantly greater tumor growth delay than treatment with radiation and TXL while both drugs were given at the same equivalent TXL dose of 60 mg/kg 24 hr after tumor irradiation (enhancement factors: 4.44 vs. 1.50). These data suggest that conjugation of TXL to poly(L-glutamic acid) is necessary for improved response, and that the supra-additive effect of combined radiation and PG-TXL therapy is in part due to modulation of the enhanced permeability and retention effect of macromolecules by radiation.

The effect of time interval between tumor irradiation and injection of PG-TXL was investigated using mammary MCa-4 tumor (4). Tumors irradiated at 1-24 h prior to injection of PG-TXL resulted in significant tumor growth delay, yielding enhancement factor of about 2.0 in this model. Interestingly, combined radiation and free TXL yielded enhancement factor of 0.86 to 1.0, indicating that only additive or even sub-additive interactions were produced when radiation preceded TXL treatment. This observation is in agreement with in vitro findings reported in the literature that suggest radiation could antagonize the cytotoxicity of TXL (5, 6). Prior radiation can modify the effect of subsequent TXL treatment by inducing cell cycle delay. It is possible that by using a polymeric conjugate such as PG-TXL, the effect of altered cell cycle caused by radiation is reduced to minimum because the drug persisted in the tumor for a longer period of time.

To examine whether the superior synergistic interaction between PG-TXL and radiation also exist when PG-TXL is given prior to tumor irradiation, C3Hf/Kam mice bearing 8-mm murine OCa-1 tumors were treated with i.v. injected PG-TXL followed by single doses of local radiation at various time intervals. We found that PG-TXL strongly potentiated the radioresponse of OCa-1 tumor (7). The enhancement factors ranged from 2.79 to 4.28 depending on radiation dose and the interval between PG-TXL administration and radiation delivery; with greater enhancement been observed when the interval was decreased. The percentage of G2/M cells was significantly increased to 21.4% 48 h after PG-TXL, but declined to preinjection level of 14.8% 72 h after PG-TXL. PG-TXL only moderately increased the tumor vascular volume by 37% 24 h after PG-TXL administration. When compared to literature data obtained from the same tumor model, PG-TXL exhibited stronger radiosensiization effect than TXL. Although its action is possibly mediated by arrest of cells in G2/M phases of cell cycle and by increased tumor blood supply, PG-TXL may exert its radiopotentiation activity through increased tumor uptake of PG-TXL and sustained release of TXL in the tumor.

To determine if PG-TXL-induced enhancement is obtained in a more clinically relevant setting, we investigated PG-TXL effects in combination with fractionated radiation doses

and used tumor cure as the end point (8). Mice bearing 7-mm-diameter ovarian carcinomas were treated with PG-TXL at an equivalent paclitaxel dose of 80 mg/kg, single dose or 5 daily fractions of radiation or both PG-TXL and radiation. Treatment endpoint was TCD_{50} (radiation dose yielding tumor control in 50% of mice). Acute radioresponse of jejunum, skin, and hair was determined for all treatments. PG-TXL dramatically improved tumor radioresponse, reducing TCD_{50} of single-dose irradiation from 53.9 (52.2–55.5) Gy to 7.5 (4.5–10.7) Gy, an enhancement factor (EF) of 7.2. The drug improved the efficacy of fractionated irradiation even more, reducing the TCD_{50} of 66.6 (62.8–90.4) Gy total fractionated dose to only 7.9 (4.3–11.5) Gy, for an EF of 8.4. PG-TXL did not affect normal tissue radioresponse resulting from either single or fractionated irradiation. To the best of our knowledge, PG-TXL increased the therapeutic ratio of radiotherapy more than that previously reported for any other chemotherapeutic agent. PG-TXL has a high potential to improve clinical radiotherapy.

To investigate the phenomenon of increased blood permeability after radiotherapy, we have develop a macromolecular magnetic resonance imaging (MRI) agent which has paramagnetic metal ion gadolinium chelated to poly (L-glutamic acid) (9). This agent is currently under evaluation and will be used in the future for noninvasive imaging of the effect of combined radiotherapy and chemotherapy.

KEY RESEARCH ACCOMPLISHMENTS

1. We have demonstrated that PG-TXL dramatically enhances the effect of irradiation when given either before or after local tumor irradiation. An enhancement factor of greater than 4 could be achieved when tumor-growth-delay was used as the treatment end point. An enhancement factor of up to 8 was achieved when tumor-cure was used as the treatment endpoint. To our knowledge, PG-TXL increased the therapeutic ratio of radiotherapy more than that previously reported for other taxanes, thus, PG-TXL has a high potential to improve clinical radiotherapy.

2. Potentiation of antitumor response with PG-TXL after single-dose or fractionated irradiation was achieved without affecting acute normal tissue injury.

3. The mechanism of action of PG-TXL's radiosensitizing effect is multi-factorial. Modulation of the enhanced permeability and retention effect of polymers, increased tumor reoxygenation, and redistribution of cells in different phases of cell cycle may all partly contribute to the observed phenomenon.

4. On the basis of our promising results, a Phase I/II clinical trial of PG-TXL in combination with radiotherapy for the treatment of non-small lung cancer has been initiated at M.D. Anderson Cancer Center.

REPORTABLE OUTCOMES

Paper in Peer Reviewed Journals:

1. **C. Li,** S. Ke, Q-P. Wu, W. Tansey, N. Hunter, L. M. Buchmiller, L. Milas, C. Charnsangavej, S. Wallace. Potentiation of ovarian OCA-1 tumor radioresponse by poly(L-glutamicd acid)-paclitaxel conjugate. Int. J. Radiation Oncology Biol. Phys., 48: 1119-1126, 2000.
2. **C. Li,** S. Ke, Q-P. Wu, W. Tansey, N. Hunter, L. M. Buchmiller, L. Milas, C. Charnsangavej, S. Wallace. Tumor irradiation enhances the tumor-specific distribution of poly(l-glutamic acid) conjugated paclitaxel and its antitumor efficacy. Clin. Cancer Res., 6: 2829-2834, 2000.
3. Ke, S., Milas, L., Charnsangavej, C., Wallace, S., **Li, C.** Potentiation of radioresponse by polymer-drug conjugates. *J. Controlled Rel., 74:* 237-242, 2001.
4. Milas, L., Mason, K. A., Hunter, N., **Li, C.** Wallace, S. Poly(L-glutamic acid)-paclitaxel conjugate is a potent enhancer of tumor radiocurability. *Int. J. Radiation Oncology Biol. Phys.*, 55: 707-712, 2003.

Abstracts:

Li, C., Ke, Milas, L., Charnsangavej, C., and Wallace S. Sensitization of macromolecular chemotehrapy: modulation of EPR effect by radiation. 27th International Symposium on Controlled Release of Bioactive Materials, Paris, France, July 7-13, 2000.

Ke, S., Charnsagavei, C., Wallace, S., **Li, C.** Elevated Serum VEGF level as a prognostic marker in combined PG-TXL (CT-2103) and radiation therapy in mice with murine ovarian OCA-1 tumor, 92nd American Association for Cancer Research Annual Meeting March 24-28, New Orleans, LA, 2001.

List of Personnel Received Pay from the Research Effort

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CONCLUSIONS

We observed remarkable antitumor effect for the combined treatment of PG-TXL and radiation compared with the individual treatments as well as in comparison with other *in vivo* data for combined chemo and radiation therapy. Our results support the hypotheses that polymer-drug conjugates have a significant benefit when used as a novel method for delivering chemotherapy as a radiosensitizer. Our preclinical data suggest a large potential benefit of polymeric paclitaxel preparation compared with standard chemotherapy in the clinical delivery of combined chemotherapy and radiotherapy.

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distribution of poly(l-glutamic acid) conjugated paclitaxel and its antitumor efficacy. *Clin. Cancer Res.*, 6: 2829-2834, 2000.

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APPENDICES

1. Reprint: C. Li, S. Ke, Q-P. Wu, W. Tansey, N. Hunter, L. M. Buchmiller, L. Milas, C. Charnsangavej, S. Wallace. Potentiation of ovarian OCA-1 tumor radioresponse by poly(L-glutamicd acid)-paclitaxel conjugate. Int. J. Radiation Oncology Biol. Phys., 48: 1119-1126, 2000.

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BIOLOGY CONTRIBUTION

POTENTIATION OF OVARIAN OCA-1 TUMOR RADIORESPONSE BY POLY (L-GLUTAMIC ACID)-PACLITAXEL CONJUGATE

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Purpose: It has been shown that paclitaxel (TXL) can strongly enhance tumor cells' sensitivity to radiation. We examined whether the radiosensitizing effect of paclitaxel can be further enhanced when it is delivered systemically as a polymer-drug conjugate that provides enhanced tumor uptake and prolonged release of TXL in the tumor.

Methods and Materials: C3Hf/Kam mice bearing 8-mm murine ovarian OCa-1 tumors were treated with i.v.-injected Poly(L-glutamic acid)-paclitaxel (PG-TXL) at an equivalent TXL dose of 80 mg/kg, followed 24 h later by single doses of local radiation ranging from 5 to 15 Gy. To determine how long the radiopotentiation persisted at extended times after PG-TXL administration, mice with OCa-1 tumors were given i.v. PG-TXL and 4, 24, 48, 72, 120, or 168 h later their tumors were irradiated at a dose of 10 Gy. Antitumor activity was determined by delay in tumor growth. Cell cycle distribution was assayed using flow cytometry. Tumor vascular volume was estimated using Tc-99 m-labeled red blood cells.

Results: PG-TXL strongly potentiated the radioresponse of the OCa-1 tumor. The enhancement factors ranged from 2.79 to 4.28, depending on radiation dose, when PG-TXL preceded radiation by 24 h. The enhancement factor derived from radiation dose–response curves was as high as 5.13. The radiosensitizing effect of PG-TXL was also dependent on the interval between PG-TXL administration and radiation delivery, with greater enhancement been observed when the interval was decreased. The percentage of G2/M cells was significantly increased to 21.4% 48 h after PG-TXL but declined to a preinjection level of 14.8% 72 h after PG-TXL. PG-TXL only moderately increased the tumor vascular volume by 37% 24 h after PG-TXL administration.

Conclusion: PG-TXL markedly potentiated response of OCa-1 tumor to radiation. When compared to literature data obtained from the same tumor model used here, PG-TXL exhibited stronger radiosensitization effect than TXL. Although its action is possibly mediated by arrest of cells in G2/M phases of cell cycle and by increased tumor blood supply, PG-TXL may exert its radiopotentiation activity through increased tumor uptake of PG-TXL and sustained release of TXL in the tumor. Our results show that conjugation of TXL to a polymer has the potential to further enhance its radiosensitizing activity and that clinical trials of PG-TXL in combination with radiation is warranted. © 2000 Elsevier Science Inc.

Paclitaxel, Poly(L-glutamic acid)-paclitaxel, Radiosensitization, Radiotherapy, Combination therapy.

INTRODUCTION

Paclitaxel (Taxol, TXL) is a potent natural microtubule-targeting antitumor product isolated from the bark of the pacific yew, *Taxus brevifolia* (1). When tumor cells are exposed to cytotoxic amounts of TXL for hours or days, the cells are arrested in the G2/M phase of the cell cycle in large proportion (2, 3). Based on this observation and the radiobiological principle that G2 and M are the most radiosensitizing phases of the cell cycle (4), a combination of TXL with ionizing radiation has been proposed. Pioneering studies showed a significant interaction between TXL and radiation, and the extent of the interaction was dependent on the concentration and duration of TXL exposure (5, 6). Further studies showed that enhanced radiation cell killing by TXL does not work in all ranges of drug concentrations. Hennequin *et al.*, for instance, showed that brief, concomitant exposure to TXL or docetaxel in two cell lines reduces radiation-induced cell killing when the drug concentrations are below certain threshold levels (7). Zanelli *et al.* showed that maximum radiosensitization is not achieved unless the drug has been in contact with the cells for approx-

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imately 24 h and that the radiosensitization effect is lost quickly soon after the drug is removed (8). Thus, it appears that the concentration and the resident time of TXL in the tumor may be important factors in determining the extent of interaction between TXL and radiation. Insufficient tumor deposition of the drug could potentially induce radiation resistance.

After i.v. administration to mice, TXL was rapidly cleared from the blood, with a half-life ($t_{1/2}$) of 29 to 69 min; the drug was distributed extensively to all tissues except the brain and testicle (9). Based on *in vitro* findings and pharmacokinetic calculations, Zanelli *et al.* suggest a low-dose, daily infusion of TXL for as long as possible during radiotherapy (8). The proposed protocol, however, has not been validated in an animal model. In addition to technical difficulties, a major concern is the potential systemic toxicity associated with daily administration of TXL. Instead, most animal studies performed to date have used treatment protocols in which single bolus i.v. injections of conventionally formulated TXL were administered. Under these circumstances, TXL has been found to enhance tumor radioresponse, with enhancement factors ranging from 1.2 to 2.5, the values of which depend on the tumor type, the dose of radiation, and the time between TXL administration and radiation delivery (10, 11).

One approach that can potentially provide prolonged tumor exposure to TXL is to use a drug delivery system that offers desirable pharmacokinetic properties. Polymer-drug conjugates have been investigated as carriers for anticancer drugs in an attempt to direct active agents to tumors and to reduce toxic effects to normal tissues (12–15). Because of increased vascular permeability, tumors tend to take up polymers more readily than do normal tissues. Furthermore, polymeric drugs are retained in the tumor due to the lack of lymphatics (12). We have recently reported the synthesis and antitumor activity of a water-soluble poly(L-glutamic acid)-TXL conjugate, PG-TXL (14). PG-TXL exhibited markedly greater antitumor activity against murine tumors and human tumor xenografts than TXL (14, 15). The biodistribution of PG-TXL in mice with OCa-1 is notable: total exposure to TXL from the time of administration to 144 h after administration is five times greater when administered as PG-TXL as compared to TXL formulated in cremophor/alcohol vehicle (16). Furthermore, TXL is released from PG-TXL gradually, and significant concentrations of free TXL are maintained in the tumor for up to 144 h (16). In the present study, we explored whether PG-TXL could offer stronger enhancement of tumor radioresponse.

METHODS AND MATERIALS

TXL was obtained from Hande Tech (Houston, TX). PG-TXL (molecular weight, 36,000–49,000, TXL loading 26.8%) was synthesized according to previously described procedures (14). The conjugate was dissolved in saline to an equivalent TXL concentration of 8 mg/ml and filtered through a 0.22-μm sterile filter before injection.

Mice and tumors

Female C3Hf/Kam mice (25–30 g) were bred and maintained in a specific pathogen-free mouse colony in the Department of Experimental Radiation Oncology at our institution. All experiments involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Solid murine ovarian OCa-1 tumors were generated in the muscles of the right thighs of mice by inoculating 5×10^5 viable tumor cells in suspension in phosphate-buffered saline (PBS). The OCa-1 tumor originally developed spontaneously and was syngeneic to this strain of mice.

Tumor response to treatments

The antitumor effects of PG-TXL and radiation, alone and in combination, were determined by their ability to delay tumor growth. When tumors had grown to 8 mm in average diameter, mice were randomly allocated into groups, with each group typically consisting of six mice. Local treatment to the tumor with graded single doses of 5–15 Gy irradiation was delivered with a ^{137}Cs source at a dose rate of 6.25 Gy/min. The mice were immobilized on a jig during irradiation, and the tumor was centered in the circular irradiation field 3 cm in diameter. We had previously found that a single i.v. dose of PG-TXL injected at an equivalent TXL dose of 160 mg/kg caused complete regression of OCa-1 tumors (14). Preliminary study combining PG-TXL at an equivalent TXL dose of 120 mg/kg and 15 Gy also showed complete tumor control. Therefore, to avoid tumor elimination but still achieve measurable tumor regrowth, an equivalent TXL dose of 80 mg/kg PG-TXL was chosen. In the combined PG-TXL and radiation therapy, PG-TXL was administered 4–168 h before the radiation. Tumor growth was determined daily by measuring three orthogonal tumor diameters. The mice were killed when tumors reached 14 mm in diameter.

Flow cytometry analysis of cell cycle

The procedure to isolate cells from frozen solid mass was performed according to Dressler *et al.* (17). OCa-1 tumors were collected 4, 24, 48, and 72 h after i.v. injection of PG-TXL at an equivalent TXL dose of 80 mg/kg, or 4 and 24 h after TXL at an i.v. dose of 40 mg/kg. Control mice were not treated. For flow cytometric analysis, frozen tumor was transferred to a Petri dish containing 10 mL of basic minimal essential medium with 5% fetal bovine serum (FBS). Cells were released by cutting the tumor in half and scraping the surface. The cell suspension was filtered through 53-μm nylon mesh (Spectrum, Laguna Hills, CA) and washed using culture medium containing 5% FBS. The cell concentration was then adjusted to 1×10^6 to 3×10^6 cells/ml and stained with fluorochrome buffer (0.1% sodium citrate, 0.3% Triton X-100, 20 μg/ml RNase, 50 μg/ml propidium iodide) in the dark at 4°C overnight. The cellular content was analyzed using a Coulter SL flow cytometer (Coulter, Miami, FL) after the cells were filtered through a 37-μm nylon mesh (Spectrum).

Table 1. Effect of PG-TXL on radioresponse of OCa-1 tumor as a function of radiation dose

Treatment*	Time in days for tumors to grow from 8–12 mm (mean ± SE)	Absolute growth delay†	Normalized growth delay‡	Enhancement factor§ (95% C.I.)
Saline	12.8 (±0.7)			
PG-TXL (80 mg/kg)	29.4 (±4.4)	16.6		
5 Gy	18.5 (±1.3)	5.7		
10 Gy	24.0 (±1.6)	11.2		
15 Gy	28.5 (±2.1)	15.7		
PG-TXL + 5 Gy	45.3 (±5.0)	32.5	15.9	2.79 (0.54, 5.09)
PG-TXL + 10 Gy‖	57.2 (±8.1)	44.4	27.8	2.48 (0.47, 4.51)
PG-TXL + 15 Gy	96.6 (±8.0)	83.8	67.2	4.28 (2.92, 5.65)

* Mice were given PG-TXL at an equivalent paclitaxel dose of 80 mg/kg as a single i.v. injection. When PG-TXL and irradiation were combined, local irradiation was delivered 24 h after PG-TXL treatment. The groups consisted of 6 mice each.

† Absolute growth delay was defined as the time in days for tumors in treated groups to grow from 8 to 12 mm minus the time in days for tumors in the saline-treated control group to grow to the same size.

‡ Normalized growth delay was defined as the time in days for tumors to grow from 8 to 12 mm in mice treated with the combination of PG-TXL and radiation minus the time in days for tumors to grow to the same size in mice treated with the same dose of PG-TXL alone.

§ Enhancement factors were obtained by dividing normalized tumor growth delay by the absolute growth delay in mice treated with radiation only.

‖ Analysis was based on five mice; one of six mice had total tumor control at 120 days after the treatment.

Tc-99m-red blood cell estimates of vascular volume

Tc-99m-labeled red blood cells (RBC) were prepared according to the manufacturer's instructions (Ultra Tag RBC kit, Mallinckrodt Medical, St. Louis, MO). Half-milliliter of C3Hf/Kam mice blood freshly collected in a tube containing anticoagulant heparin was transferred to a vial containing stannous chloride and allowed to react for 5 min. Sodium hypochloride (0.6 mL) and a citrate-buffered solution (pH 5.0, 1.0 mL) were then added to the vial. Sodium pertechnetate Tc-99 m (70 μCi) was added to the reaction vial, and the mixture was allowed to react for 20 min. The labeling efficiency of RBC was 97%. Mice were injected i.v. with 0.2 mL of the Tc-99m-labeled RBC (5 μCi) and killed 30 min later. Samples of blood and tumor were obtained, weighed, and counted for radioactivity in a Packard γ-counter (Downers Grove, IL). To prevent blood from being lost in the specimens, tissues were removed after blood circulation was stopped. The vascular volume of the tumors was calculated as:

$$\textit{Vascular Volume } (\mu L/g \textit{ tissue}) = (\textit{radioactivity/g tumor})/(\textit{radioactivity/}\mu L \textit{ blood}).$$

Data analysis

The effect of treatment on tumor growth was expressed as the absolute growth delay, which was defined as the time in days for tumors in treated mice to grow from 8 mm to 12 mm minus the time in days for tumors in the control saline-treated mice to reach the same size.

The Student's *t* test was used to compare differences in tumor vascular volumes and differences in percentages of cells in different phases of cell cycle between taxane-treated groups and control group.

RESULTS

Radiosensitizing effect of PG-TXL

To determine whether PG-TXL enhances tumor response to radiation *in vivo*, mice bearing 8-mm diameter OCa-1 tumors were divided into experimental groups and treated as follows: saline control, PG-TXL, radiation, and PG-TXL plus radiation delivered 24 h later. Single doses of radiation, ranging from 5 to 15 Gy, were delivered locally to the tumor. Tumor growth was delayed by PG-TXL alone and by radiation alone in a dose-dependent manner. When PG-TXL was combined with radiation, tumor growth delay increased by 16, 28, and 67 days, yielding enhancement factors of 2.79, 2.48, and 4.28 at radiation doses of 5 Gy, 10 Gy, and 15 Gy, respectively (Table 1). To illustrate the magnitude of the antitumor efficacy of the combined treatment, tumor growth curves were plotted for mice treated with PG-TXL at an equivalent TXL dose of 80 mg/kg, for mice treated with 15 Gy radiation, and for mice treated with PG-TXL plus radiation with 24 h intervals between treatments (Fig. 1). To quantify the extent of PG-TXL-induced tumor radiopotentiation, the values of absolute tumor growth delay produced by radiation in PG-TXL-treated mice or untreated mice are plotted as a function of radiation dose (Fig. 2). PG-TXL treatment shifted the radiation dose response curve to the left, indicating synergistic interaction between these two modalities. The enhancement factor representing the ratio of isoeffective radiation doses was 5.13.

To determine how long the radiopotentiation persisted at extended times after PG-TXL administration, mice bearing 8-mm tumors were given i.v. PG-TXL, and 4, 24, 48, 72, 120, or 168 h later their tumors were locally irradiated with a single radiation dose of 10 Gy. Table 2 summarizes the effect of PG-TXL on radioresponse of OCa-1 tumor as a function of time interval between PG-TXL administration

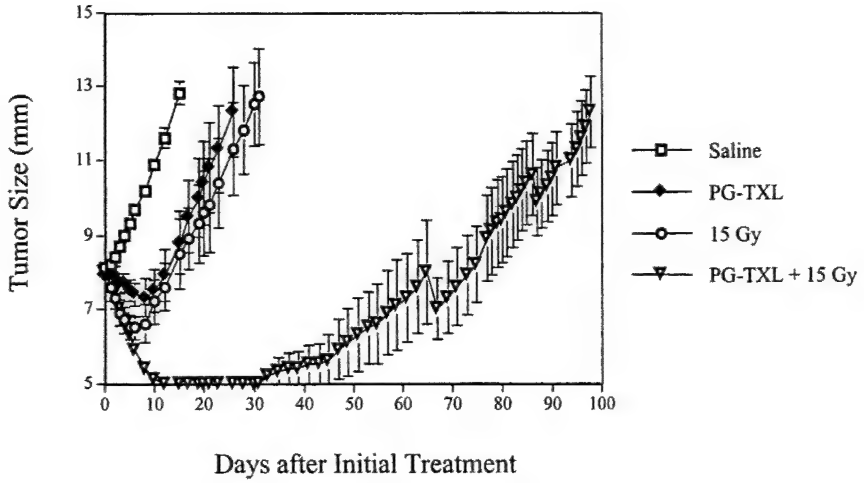

Fig. 1. Effect of PG-TXL and radiation on the growth of OCa-1 tumors in mice. Mice bearing 8-mm tumors in the right hind leg were given (open box) saline, (filled diamond) PG-TXL as a single i.v. injection at an equivalent paclitaxel dose of 80 mg/kg, (open circle) 15 Gy of local tumor irradiation, or (open triangle, pointing down) a single i.v. injection of PG-TXL plus 15 Gy radiation 24 h later. Each data point represents the mean size of six tumors; bars, SE. The vertical axis is labeled from 5 mm in tumor diameter because accurate measurement of tumor size was not possible below this size.

and radiation delivery. All treatment groups combining PG-TXL with local irradiation significantly delayed tumor growth as compared to treatment with 10 Gy irradiation alone ($p < 0.02$). Furthermore, when delivered within a time interval of 72 h, PG-TXL potentiated the radiation response of OCa-1 tumor; the enhancement factors were 3.69, 2.59, 2.51, and 1.97 respectively when PG-TXL was given at 4, 24, 48, and 72 h before tumor irradiation. An additive effect was observed when PG-TXL preceded irradiation by 120 h or longer (Table 2). It should be noted that combined treatments at 4 h and 24 h intervals caused permanent tumor control in some mice.

PG-TXL arrests tumor cells in G2/M phase

To determine whether PG-TXL increased cellular sensitivity to radiation through cell cycle redistribution, we analyzed the OCa-1 tumor cells at different times after PG-TXL treatment by flow cytometry. Treatment with PG-TXL at an equivalent TXL dose of 80 mg/kg increased the percentage of G2/M cells from 14.6% to 21.4% (p =

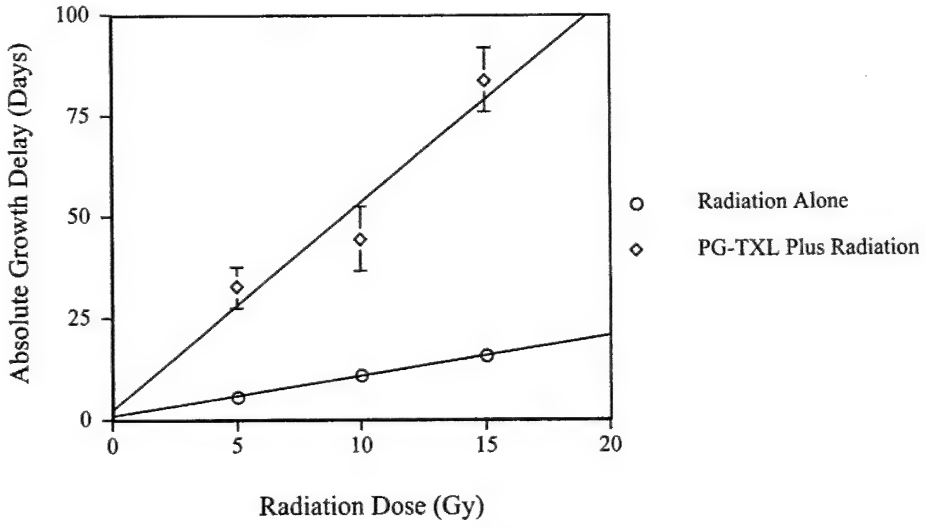

Fig. 2. Effect of PG-TXL on radioresponse of OCa-1 tumor growth as a function of radiation dose. Mice bearing 8-mm tumors in the right thigh were given 5, 10, or 15 Gy of local tumor radiation alone or i.v. PG-TXL (80 mg/kg equivalent paclitaxel) followed 24 h later by the same doses of radiation. Groups consisted of six mice each. Vertical bars are SE. Data were fitted to straight lines by linear regression. The enhancement factor of 5.13 is represented by the ratio of the slope of the combined modality treatment to the slope of the radiation-only treatment.

Table 2. Effect of PG-TXL on radioresponse of OCa-1 tumor as a function of time interval between PG-TXL administration and radiation delivery

Treatment*	Time in days for tumors to grow from 8–12 mm (mean ± SE)	Absolute growth delay	Normalized growth delay	Enhancement factor (95% C.I.)
Saline	10.8 (±0.6)			
PG-TXL (80 mg/kg)	20.5 (±2.7)	9.7		
10 Gy	22.4 (±0.7)	11.6		
PG-TXL + 10 Gy (4 hr)†	63.3 (±19.9)	52.5	42.8	3.69 (0.33, 7.05)
PG-TXL + 10 Gy (24 hr)‡	50.6 (±9.7)	39.8	30.1	2.59 (0.96, 4.23)
PG-TXL + 10 Gy (48 hr)	49.6 (±5.2)	38.8	29.1	2.51 (1.64, 3.38)
PG-TXL + 10 Gy (72 hr)	43.3 (±8.8)	32.5	22.8	1.97 (0.48, 3.46)
PG-TXL + 10 Gy (120 hr)	31.2 (±3.6)	20.4	10.7	0.92 (0.32, 1.53)
PG-TXL + 10 Gy (168 hr)	32.6 (±4.0)	21.8	12.1	1.04 (0.36, 1.72)

* Mice were given PG-TXL at an equivalent paclitaxel dose of 80 mg/kg as a single i.v. injection. When PG-TXL and irradiation were combined, local irradiation was delivered at different times after PG-TXL treatment. The groups consisted of 5–6 mice each. Refer to Table 1 for definition of absolute growth delay and enhancement factor.

† Analysis was based on three mice; two of five mice had total tumor control at 120 days after the treatment.

‡ Analysis was based on four mice; one of five mice had total tumor control at 120 days after the treatment.

0.0004) 48 h after drug injection. By 72 h, the percentage of cells in the G2/M phase declined to the preinjection level (Table 3). Interestingly, cells in S-phase decreased significantly 72 h after PG-TXL treatment ($p = 0.039$). Percentages of cells in phases of cell cycle for tumors collected at 4 and 24 h after PG-TXL treatment were not obtained due to massive cellular death (sub G_0/G_1 population > 85%). The effect of TXL treatment on cell cycle redistribution was also analyzed. TXL caused significant increase in the population of G2/M cells at 4 h ($p = 0.0021$) and 24 h ($p = 0.026$) (Table 3).

Effect of PG-TXL on functional tumor vascular volume

Tumor reoxygenation has been shown to be an important mechanism by which TXL potentiates the radioresponse of tumors *in vivo* (18). To determine whether treatment with PG-TXL increased the blood supply to the tumor and thus presumably tumor oxygenation as well, Tc-99m-RBC was used to estimate the functional vascular volume of OCa-1 tumors of 8 mm in diameter. The vascular volume was 9.45 μL/g tumor at 4 h after PG-TXL injection, which was not statistically different from the control value of 8.95 μL/g. A moderate increase in vascular volume (12.3 μL/g tumor, $p = 0.057$) was observed at 24 h. Thus, the vascular supply of nutrients and oxygen was 37% higher 24 h after PG-TXL injection than it was without PG-TXL treatment.

Table 3. Percentage of cells in various phases of cell cycle after taxane treatments

Treatment*	G1	G2/M	S
Control	57.9 (±3.1)	14.6 (±1.0)	27.8 (±3.5)
PG-TXl (48 h)	55.9 (±2.8)	21.4 (±1.3)†	22.7 (±3.8)
PG-TXL (72 h)	65.4 (±0.3)	14.8 (±0.6)	19.8 (±0.8)‡
TXL (4 h)	55.1 (±2.6)	22.4 (±2.5)†	22.5 (±5.0)
TXL (24 h)	56.8 (±2.5)	17.4 (±0.8)‡	25.9 (±1.6)

* PG-TXL was given i.v. at an equivalent TXL dose of 80 mg/kg; TXL was given i.v. at a dose of 40 mg/kg. The groups consisted of 3 mice each.

† Highly significant difference found between the treatment group and the control group by Student's *t*-test, $p < 0.005$.

‡ Significant difference found between the treatment group and the control group by Student's *t*-test, $p < 0.05$.

DISCUSSION

In the past, much attention has been given to identifying new chemotherapeutic drugs with radiosensitizing properties and to optimizing treatment schedules and radiation dosage. Few studies have investigated the role of sustained drug delivery to the tumor in achieving effective therapeutic combinations (19, 20). Williams *et al.* showed that incorporation of iododeoxyuridine (IdUrd) into biodegradable polyanhydride copolymers and intratumoral delivery of the polymer implants into a human glioma tumor inoculated in nude mice resulted in more effective tumor control than radiation alone (19). Similarly, Yapp *et al.* showed that cisplatin/polyanhydride implants delivered intratumorally enhanced the cisplatin concentration in the tumor and that this system of drug delivery more effectively potentiated the effect of both acute and fractionated radiation than systemic cisplatin administration (20). These studies suggest that the sustained release of chemotherapeutic agents enhances the effects of sensitization during the critical period of radiotherapy.

The method of intratumoral administration, however, is limited to tumors that are accessible to implantation. More important, the ability of chemotherapeutics in controlling systemic metastases is minimized when systemic administration was not used. Water-soluble polymer-drug conjugates, on the other hand, can be administered systemically and are capable of selectively delivering active drugs to the tumor due to increased tumor vascular permeability and the

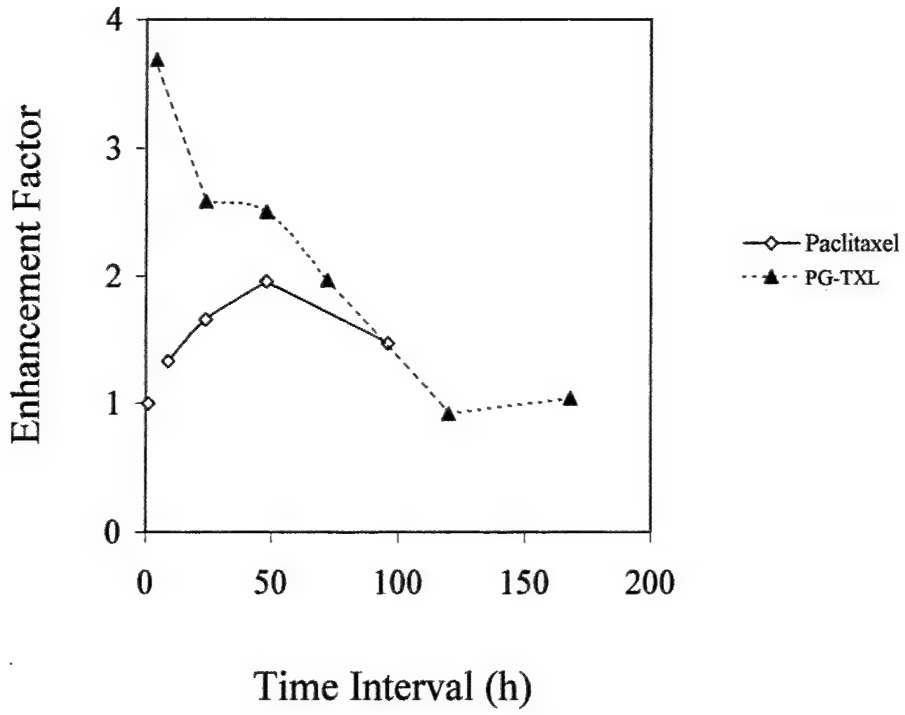

Fig. 3. Comparison of the effect of interval between administration of taxane and radiation delivery on the enhancement factor when taxanes are combined with local tumor irradiation. Data for paclitaxel are taken from reference 11, where paclitaxel was injected i.v. at a dose of 40 mg/kg, and radiation was delivered at a dose of 14 Gy. Data for PG-TXL are obtained from this study. PG-TXL was injected at an equivalent paclitaxel dose of 80 mg/kg, and radiation was delivered at a dose of 10 Gy.

retention effect of polymers in the tumor. In the present study, PG-TXL was chosen as a model compound to test whether a water-soluble polymer-TXL conjugate injected i.v. produces increased interaction with radiation. It has been previously shown that i.v. PG-TXL delivers five times more TXL to an OCa-1 tumor than conventionally formulated TXL and that PG-TXL acts as a depot for sustained release of TXL within the tumor (16).

The data presented here clearly demonstrated the advantages of polymeric drug delivery system. When PG-TXL preceded tumor irradiation by 24 h, markedly enhanced response of the OCa-1 tumor to radiation was observed (Table 1 and Fig. 1). Radiation enhancement factors derived from tumor growth delay ranging from 2.48 to 4.28 were obtained, depending on the dose of radiation. The enhancement factor generated from the radiation dose response curves was 5.13 (Fig. 2). These values were much higher than values obtained for TXL under the similar conditions using the same animal model. When TXL at a dose of 40 mg/kg was given 24 h before 14 Gy of radiation, an enhancement factor of 1.66 was obtained (11). Likewise, an enhancement factor of 1.97 was derived from the radiation dose response curves when TXL at a dose of 40 mg/kg was injected 24 h before tumor irradiation (11). Thus, by simply changing the biodistribution and pharmacokinetics of TXL through the use of polymeric conjugate, substantial improvement in the drug's radiosensitizing activity was achieved.

The interval between PG-TXL and radiation administration was another major factor influencing the degree of radiopotentiation. When given 4, 24, 48, and 72 h before irradiation, PG-TXL and radiation produced supra-additive effect. The effect was smaller as the time intervals between PG-TXL and radiation were increased (Table 2). By 120 h, PG-TXL and radiation produced only additive effect. To compare the extent and kinetics of radiosensitizing effect between PG-TXL and TXL, the enhancement factors produced by PG-TXL (data from this study) and TXL (data from reference 11) were plotted as a function of time intervals between delivery of taxane and radiation (Fig. 3). PG-TXL not only produced a stronger radiopotentiation effect than TXL at each time point within 48 h, the kinetics of radiopotentiation effect of PG-TXL is also different from that of TXL. When TXL was combined with radiation, the enhancement effect increased as the interval increased from 9 to 48 h, then decreased at longer interval (11).

Flow cytometry was used to analyze the distribution of tumor cells at different phases of the cell cycle. As expected, TXL treatment caused significant increase in the population of G2/M cells to 22.4% at 4 h and 17.4% at 24 h. PG-TXL also caused significant increase in the percentage of G2/M cells to 21.4% at 48 h (Table 3). Although data were not obtained for 4 h and 24 h time points in the present study, we have previously shown that PG-TXL at an equivalent TXL dose of 160 mg/kg caused increased mitotic arrest of OCa-1 tumor cells at these time intervals by

histologic analysis (14). Thus, enhanced radioresponse of OCa-1 tumor at 4–48 h after PG-TXL treatment may be attributed in part to the arrest of tumor cells in the G2/M phase. However, our data do not support that G2/M arrest being the major mechanism responsible for PG-TXL's strong radiosensitizing activity for the following considerations. First, the percentage of OCa-1 cells in G2/M declined to the pretreatment level by 72 after PG-TXL. At this time interval, the radiopotentiation effect of PG-TXL was still strong, with an enhancement factor of 1.97. This is not in concordance with the *in vitro* findings that tumor cells treated with TXL exhibited radiosensitizing activity only when irradiated in G2/M (3). Second, an enhancement factor as large as 3.69 was obtained as early as 4 h after PG-TXL treatment. It is unlikely that the percentage of G2/M cells could reach such a magnitude at this time interval to produce a strong radiopotentiation effect as observed, keeping in mind the fact that only a fraction of free TXL is released in the tumor during this time. Finally, our data cannot explain why PG-TXL exhibited a stronger radiopotentiation effect than TXL.

Alternatively, the radiosensitizing effect of PG-TXL may be mediated by tumor reoxygenation, since it has been suggested that TXL potentiated tumor response to radiation *in vivo* through a complex process involving apoptosis, cell depopulation, and reoxygenation (18). To determine whether treatment with PG-TXL affected tumor blood supply and thus likely the oxygenation status of the tumor as well, we measured the functional tumor vascular space using radiolabeled RBC (21). Our data showed that the vascular volume of the tumor had increased 37% 24 h after PG-TXL treatment. However, such an increase was only moderate. Thus, although PG-TXL may enhance tumor response to radiation by increasing the vascular delivery of oxygen to the tumor at 24 h after treatment, these changes are relatively small to account for the strong radiosensitizing effect of PG-TXL observed. Furthermore, the data cannot be used to explain the radiopotentiation effect at 4 h after PG-TXL, since no significant change in tumor vascular volume was observed at this time. It should be noted, however, that other methods, such as tumor irradiation under hypoxic conditions and PO_2 measurements, need to be employed to assess more accurately the influence of PG-TXL on tumor oxygenation.

A more probable explanation is that radiation enhanced the cytotoxic effect of PG-TXL by allowing more PG-TXL get into the tumor tissue. We have previously shown that, in contrast to a number of *in vitro* findings, irradiation of OCa-1 tumor before PG-TXL administration actually results in supra-additive effect (22). We believe that such an effect is not due to increased sensitivity of tumor cells toward PG-TXL by tumor irradiation. Rather it is because of the increased tumor exposure to long-acting PG-TXL as a result of two contributing factors. First, the killing effect of radiation reduces the population of viable cells and subsequently increases the availability of each surviving tumor cell to PG-TXL. Second, radiation increases the tumor uptake of PG-TXL. These factors, combined with the retention of PG-TXL and sustained release of TXL from PG-TXL within the tumor, is likely responsible for the enhanced radiopotentiation effect of PG-TXL observed in the current studies as well. Our observation that the enhancement factors were greater when the time intervals between PG-TXL treatment and tumor irradiation became shorter supports this view. Shorter intervals imply stronger impact of the killing effect of radiation, greater influence of radiation on tumor vascular permeability toward PG-TXL, and subsequently greater antitumor activity of PG-TXL. Because of prolonged circulation time of PG-TXL in the blood, the effect of radiation manifested as enhanced tumor response could still be observed 72 h after PG-TXL injection. At longer intervals such as 120 and 168 h, the importance of radiation on the action of PG-TXL diminished.

In conclusion, we have shown that water-soluble polymeric drug conjugates may have an important role in enhancing the efficacy of radiation therapy. To our knowledge, this is the first report demonstrating that a stronger radiopotentiation activity of a radiosensitizer could be obtained by conjugating the parent drug to a polymeric carrier. Although comparison between PG-TXL and TXL in Fig. 3 may not be adequate because of difference in the doses of taxane and radiation used, it needs to be stressed that the dose of TXL (80 mg/kg) chosen for PG-TXL is highly toxic if used in the free form. Thus, our data demonstrate the advantage of PG-TXL over TXL. An important conclusion from this study and our previous finding that radiation given before PG-TXL also potentiates its antitumor activity (22) is that the effect of combined PG-TXL and radiation is not so much dependent on the sequence of treatments when delivered within 24 h with each other. But the potentiation of tumor response to radiation by PG-TXL is dependent on the time intervals between the delivery of these two modalities with shorter intervals preferred. With this in mind it would be useful to investigate the effects of combination therapy between other polymer-drug conjugates and radiation in other tumor models. These studies will help in defining the nature and extent of interaction between polymeric drugs and radiation and facilitate in transferring this promising approach to the clinical setting.

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Tumor Irradiation Enhances the Tumor-specific Distribution of Poly(L-glutamic acid)-conjugated Paclitaxel and Its Antitumor Efficacy[1]

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ABSTRACT

The poly(L-glutamic acid)-paclitaxel (PG-TXL) conjugate has been shown to exhibit significantly greater antitumor activity than conventionally formulated paclitaxel (TXL) against solid tumors (Li *et al.*, Cancer Res., *58:* 2404–2409, 1998). Here we report that local tumor irradiation enhanced the distribution of PG-TXL given 24 h later to ovarian OCa-1 carcinoma implanted i.m. in C3Hf/Kam mice. Radiation significantly increased tumor uptake of PG-TXL and tumor vascular permeability, caused elevation of the serum concentration of vascular endothelial growth factor, and arrested OCa-1 cells in the G_1 phase of cell cycle. The enhancement factors, as measured by incremental tumor growth delay compared with PG-TXL alone, ranged from 1.36–4.44. Complete tumor regression was also observed at a higher radiation dose (>10 Gy) and a higher PG-TXL dose (>80 mg equivalentTXL/kg). Furthermore, combined radiation and PG-TXL produced a significantly greater tumor growth delay than treatment with radiation and TXL when both drugs were given at the same equivalent TXL dose of 60 mg/kg 24 h after tumor irradiation (enhancement factors, 4.44 *versus* 1.50). These data suggest that conjugation of TXL to poly(L-glutamic acid) is necessary for improved response and that the supra-additive effect of combined radiation and PG-TXL therapy is due in part to modulation of the enhanced permeability and retention effect of macromolecules by radiation. We propose a treatment strategy combining radiation and macromolecular chemotherapy that may have important clinical implications in terms of scheduling and optimization of the therapeutic ratio.

INTRODUCTION

Many solid tumors have disordered capillary endothelium and are thus more permeable to macromolecules than is normal tissue (1, 2). In addition, because of the paucity of lymphatics in solid tumors, macromolecules tend to persist longer in tumors than in normal tissue. This phenomenon, called the EPR[3] effect, is thought to be responsible for the enhanced uptake of macromolecular drugs seen in solid tumors (2). Therefore, the use of water-soluble macromolecular chemotherapeutic agents is an attractive approach to minimizing the systemic toxicity and improving the therapeutic efficacy of anticancer drugs (2–4).

We recently reported a new polymer-drug conjugate, PG-TXL, that is prepared by conjugating TXL with poly(L-glutamic acid) via ester bonds (5). Compared with TXL, PG-TXL exhibits greater antitumor activity against both murine tumors and human tumor xenografts (5, 6). This finding is largely attributable to a significantly higher uptake of PG-TXL (molecular weight, 40,000) than TXL (molecular weight, 854) in tumors.[4]

Because cancer chemotherapy based on the EPR effect is becoming prominent in anticancer drug development, it is important to identify treatment modalities that may facilitate the delivery of macromolecular drugs to tumor tissue with greater selectivity. It has been shown that radiation increases the vascular permeability of solid tumors (7, 8). Moreover, tumor-secreted VEGF, also known as vascular permeability factor, has been identified as a potent vascular permeability enhancer and an angiogenesis promoter (9). These findings prompted us to hypothesize that enhancement of vascular permeability by radiation may cause enhanced tumor uptake of macromolecular drugs, leading to improved antitumor efficacy. The purpose of this study is to determine whether radiation followed by PG-TXL will produce a supra-additive antitumor effect in the treatment of murine ovarian OCa-1 tumor.

MATERIALS AND METHODS

Drugs. TXL (Hande Tech, Houston, TX) was dissolved in cremophor/alcohol (1:1, v/v) at a concentration of 30 mg/ml. The stock solution was diluted with saline (1:4, v/v) immediately before injection. PG-TXL (molecular weight, 36,000–

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[3] The abbreviations used are: EPR, enhanced permeability and retention; PG-TXL, poly(L-glutamic acid)-paclitaxel; TXL, paclitaxel; VEGF, vascular endothelial growth factor.

[4] C. Li *et al.*, manuscript submitted.

49,000; TXL loading, 12.8–26.8%) was synthesized according to procedures described previously (5). The conjugate was dissolved in saline at an equivalent TXL concentration of 8 mg/ml and filtered through a 0.22-μm sterile filter before injection.

Mice and Tumors. Female C3Hf/Kam mice (25–30 g) were bred and maintained in a specific pathogen-free mouse colony in the Department of Experimental Radiation Oncology (The University of Texas M. D. Anderson Cancer Center, Houston, TX). All experiments involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Solid murine ovarian OCa-1 tumors were generated in the muscles of the right thigh of mice by the injection of 5×10^5 viable tumor cells in suspension in PBS. The OCa-1 tumor originally developed spontaneously and was syngeneic to this strain of mice (10).

Antitumor Efficacy of Treatments with Radiation, Taxanes, and Radiation Plus Taxanes. The antitumor effects of radiation alone, taxanes alone, and combined therapy with a single i.v. injection of PG-TXL or TXL given 24 h after tumor irradiation were determined in terms of their ability to delay tumor growth. When tumors had grown to 8 mm in average diameter, mice were randomly allocated into groups of six mice. Graded single doses of 5, 10, or 15 Gy were delivered to the tumor with a ^{137}Cs source at a dose rate of 6.25 Gy/min. We have previously found that PG-TXL at an equivalent TXL dose of 160 mg/kg administered in a single i.v. dose causes complete regression of OCa-1 tumors (5). Therefore, to avoid tumor elimination and permit measurable tumor regrowth, PG-TXL at an equivalent TXL dose of 60–80 mg/kg was administered. Tumor growth was determined daily by measuring three orthogonal tumor diameters. The mice were sacrificed when tumors reached 14 mm in diameter.

Tumor Uptake of Tritium-labeled PG-TXL. The [^{3}H]PG-TXL conjugate was synthesized from [^{3}H]poly(L-glutamic acid) and TXL using a procedure described previously (5). The resulting conjugate contained 20% (w/w) TXL, and the specific activity was 2.30 μCi/mg conjugate or 11.5 μCi/mg equivalent TXL (25,535 dpm/μg). Before injection, the conjugate was dissolved in saline to an equivalent TXL concentration of 4 mg/ml. Mice with OCa-1 tumors of 8 mm in average diameter were randomly allocated into two groups of four mice each. Mice in group 1 were injected i.v. with [^{3}H]PG-TXL at an equivalent TXL dose of 20 mg/kg (8 μCi). Mice in group 2 were treated with 15 Gy delivered to the tumor, followed by an i.v. injection of the same dose of [^{3}H]PG-TXL 24 h later.

Animals were sacrificed at 5, 24, or 144 h after injection of [^{3}H]PG-TXL. Blood, kidney, liver, muscle, spleen, and tumor tissues were removed, weighed, dissected, and dissolved in tissue solubilizer (Packard, Meriden, CT) until clear solutions were obtained. Scintillation solvent was added into aliquots of the tissue solutions, and the mixtures were assayed for total radioactivity. The counting efficiency was verified by the method of standard addition. The concentration of radioactivity in each sample was expressed as dpm/g.

Quantification of Tumor Vascular Permeability. Tumor vascular permeability was measured by the Evans Blue (Sigma, St. Louis, MO) binding method as described by Wu *et al.* (11). The dye binds to albumin (molecular weight, ~70,000) after i.v. injection and is used as a marker for the EPR effect of macromolecules in solid tumors. Evans Blue solution in saline (1 mg/ml) was injected i.v. at a dose of 10 mg/kg into groups of OCa-1 tumor-bearing mice (4–8 mice/group) that were either unirradiated or had been exposed to 15 Gy of local radiation. Sixteen h later, the mice were sacrificed, and the tumors were perfused with saline solution to remove the dye-albumin complex in the vascular lumen. Tumors were removed, weighed, and immersed in 3 ml of formamide (Sigma) and then incubated at 60°C for 48 h to extract the dye. The concentration of the dye was determined spectrophotometrically at 620 nm. Skin specimens were removed and quantified for dye extravasation in the same experiment, and the data were used to normalize tumor accumulation of the dye.

Analysis of Serum Levels of VEGF. Concentrations of VEGF in mouse serum were determined using ELISAs according to the instructions of the manufacturer (Oncogene Research Products, Cambridge, MA). Briefly, blood samples (~100 μl) were collected immediately before and 48 h after tumors were irradiated with 10–15 Gy. The blood was centrifuged, and the serum was stored at −20°C. Standards and test samples were dispensed in duplicate into 96-well microtiter plates that had been precoated with polyclonal antibody directed against VEGF. After incubation at room temperature for 2 h and washing away of unbound proteins, a second VEGF-specific antibody conjugated to horseradish peroxidase was added, and the plates were reincubated. After another washing cycle, the wells were incubated with tetramethylbenzidine. The intensity of the color developed, which was proportional to the amount of VEGF present, was measured in a microplate reader (Molecular Devices, Sunnyvale, CA) at dual wavelengths of 450 and 540 nm. The concentration of VEGF in the serum samples was determined by extrapolation from the standard curve determined from the serially diluted VEGF standard.

Flow Cytometry Analysis of Cell Cycle. OCa-1 tumors were collected 24 h after the tumors were irradiated with 15 Gy and transferred to Petri dishes with 10 ml of basic MEM with 5% fetal bovine serum. Cells were released by cutting the tumor in half and scraping the surface. The cell suspension was filtered through a 53-μm nylon mesh (Spectrum Laboratories, Laguna Hills, CA) and washed with the culture medium. The cell concentration was then adjusted to $1–3 \times 10^6$ cells/ml, and cells were stained with fluorochrome buffer (0.1% sodium citrate, 0.3% Triton X-100, 20 μg/ml RNase, and 50 μg/ml propidium iodide) in the dark at 4°C overnight. The cellular content was analyzed using Coulter SL flow cytometer (Coulter, Miami, FL).

Data Analysis. Student's t test was used to compare differences in the tumor uptake of PG-TXL and Evans Blue dye and differences in the percentages of cells in various phases of the cell cycle between irradiated and nonirradiated tumors. A value of $P < 0.05$ was considered significant.

RESULTS

Tumor Irradiation Enhanced the Antitumor Activity of PG-TXL. To determine whether tumor preirradiation enhances the tumor response to PG-TXL, mice bearing 8-mm-diameter OCa-1 tumors were divided into groups of six mice each and treated with saline, radiation alone, drugs alone, or

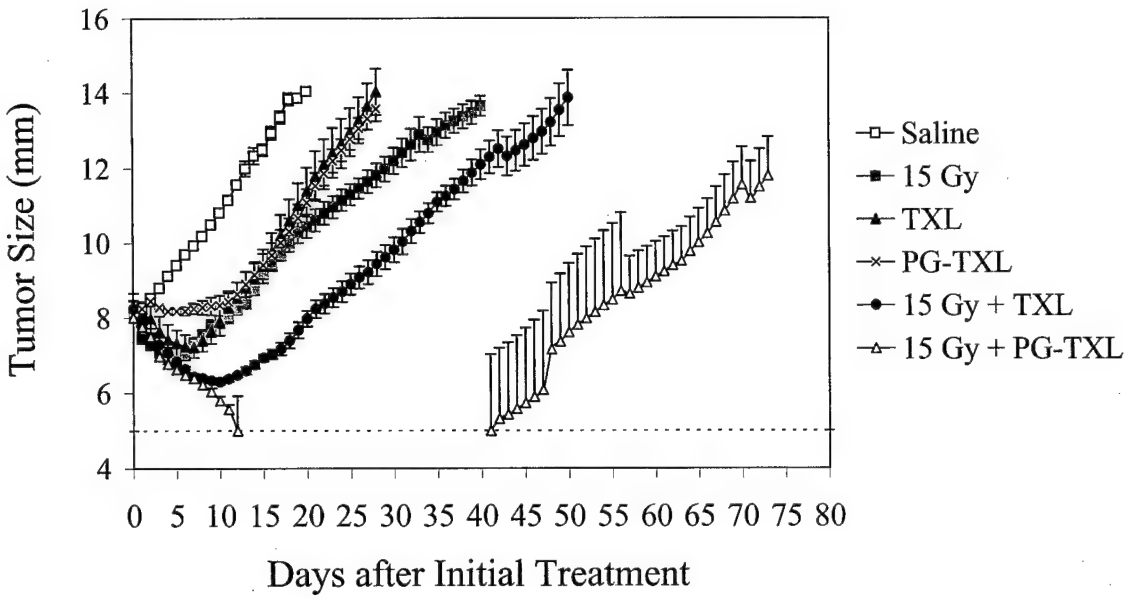

Fig. 1 Effects of combined radiation and PG-TXL and radiation and TXL on the growth of OCa-1 tumors in mice. Mice bearing 8-mm-diameter tumors in the right hind leg were given (□) saline, (▨) 15 Gy of local tumor irradiation, (▲) TXL as a single i.v. injection at an equivalent TXL dose of 60 mg/kg, (x) PG-TXL as a single i.v. injection at an equivalent TXL dose of 60 mg/kg, (●) 15 Gy of radiation plus a single i.v. injection of TXL 24 h after radiation, or (△) 15 Gy of radiation plus a single i.v. injection of PG-TXL 24 h after radiation. Each data point represents the mean size of four to six tumors; *bars*, SE. The *dotted horizontal line* represents the thigh thickness below which it is not possible to measure tumor size accurately.

radiation plus drugs. In the first experiment, mice received a single dose of 15 Gy, followed 24 h later by i.v. injection of either PG-TXL or TXL at an equivalent TXL dose of 60 mg/kg. Tumor growth delay, *i.e.*, the time in days needed for tumors to grow from 8 to 12 mm, was used as the treatment end point. Both PG-TXL and TXL at the equivalent TXL dose of 60 mg/kg were effective as single treatments. When tumors were irradiated 24 h before PG-TXL or TXL injection, the tumor growth was delayed more than the additive effect of individual treatments. However, combined radiation and PG-TXL therapy produced remarkably greater tumor growth delay than treatment with radiation and TXL (Fig. 1). The enhancement factors over the respective individual drug effect were 4.44 and 1.50 for PG-TXL and TXL, respectively (Table 1, Exp. 1).

In the second experiment, the dose response of the chemopotentiating effect of radiation was studied. Mice received single doses of radiation ranging from 5–15 Gy, followed 24 h later by i.v. injection of PG-TXL at an equivalent TXL dose of 80 mg/kg. Radiation alone produced an absolute tumor growth delay of 5.7–15.7 days when the doses were increased from 5 to 15 Gy. At a constant PG-TXL dose of 80 mg equivalent TXL/kg, the combined therapy produced an absolute tumor growth delay of 28.3–63.4 days, yielding enhancement factors of 1.37–2.87 as the radiation dose increased from 5 to 15 Gy. A total of three mice in combination groups (one at a dose of 10 Gy and two at a dose of 15 Gy) had achieved complete pathologically confirmed tumor regression (Table 1, Exp. 2).

Tumor Irradiation Increased Uptake of PG-TXL. Because radiation has been reported to increase tumor vascular permeability (8), we hypothesized that the observed increase in chemoresponse in previously irradiated tumors could be due to elevated concentrations of PG-TXL in these tumors. To determine whether prior irradiation affects the tumor uptake of PG-TXL, [^{3}H]PG-TXL was injected into mice with OCa-1 tumors 24 h after irradiation with 15 Gy. Fig. 2 shows the uptake of [^{3}H]PG-TXL in irradiated and nonirradiated tumors determined at 5, 24, and 144 h after injection of [^{3}H]PG-TXL. Irradiated tumors showed a significantly higher uptake of [^{3}H]PG-TXL than did nonirradiated tumors at all three time points. The uptake of [^{3}H]PG-TXL in irradiated tumors was 35%, 28%, and 38% higher than that in nonirradiated tumors at 5, 24, and 144 h after [^{3}H]PG-TXL injection, respectively. In contrast, no difference was found in the uptake of [^{3}H]PG-TXL in muscle tissues taken from the contralateral, unexposed thigh in irradiated and nonirradiated mice (Fig. 2). In addition, no differences were found in the concentrations of [^{3}H]PG-TXL in blood, liver, kidney, and spleen between irradiated and nonirradiated mice (data not shown). Thus, tumor irradiation increased the accumulation of PG-TXL in tumors for at least 6 days.

To determine whether increased uptake of PG-TXL was associated with increased vascular permeability, Evans Blue dye was injected into mice bearing irradiated and nonirradiated tumors, and its uptake in tumors 24 h after irradiation was quantified. The mean uptake of the dye increased from 3.32 ng/g in nonirradiated tumors to 4.18 ng/g in irradiated tumors (P = 0.025). Thus, irradiation increased tumor vascular permeability by 26%.

Serum VEGF Levels Were Elevated in Irradiated Mice. To examine whether irradiation induced a release of VEGF, we measured the concentrations of VEGF in the sera of mice before

Table 1 Effect of radiation on response of OCa-1 tumor to PG-TXL treatment as measured by tumor growth delay

Exp.[a]	Treatment[b]	Time in days for tumors to grow from 8–12 mm (mean ± SE)	Absolute growth delay[c]	Normalized growth delay[d]	Enhancement factor[e] (95% CI)
Exp. 1	Saline	14.0 ± 0.4			
	PG-TXL	23.0 ± 1.2	9.0 ± 1.2		
	TXL	22.0 ± 3.8	8.0 ± 3.8		
	15 Gy	29.0 ± 2.0	15.0 ± 2.0		
	15 Gy + PG-TXL	69.0 ± 6.5	55.0 ± 6.5	40.0 ± 6.5	4.44 (1.42–6.52)
	15 Gy + TXL	41.0 ± 2.9	27.0 ± 2.9	12.0 ± 2.9	1.50 (0.74–2.28)
Exp. 2	Saline	12.8 ± 0.7			
	PG-TXL	29.4 ± 4.4	16.6 ± 4.4		
	5 Gy	18.5 ± 1.3	5.7 ± 1.3		
	10 Gy	24.0 ± 1.6	11.2 ± 1.6		
	15 Gy	28.5 ± 2.1	15.7 ± 2.1		
	5 Gy + PG-TXL	41.2 ± 3.4	28.3 ± 3.4	22.6 ± 3.4	1.36 (0.85–1.89)
	10 Gy + PG-TXL[f]	63.0 ± 7.8	50.2 ± 7.8	39.0 ± 7.8	2.35 (0.70–3.73)
	15 Gy + PG-TXL[g]	76.3 ± 11.0	63.4 ± 11.0	47.7 ± 11.0	2.87 (0.77–4.99)

[a] Exp. experiment.

[b] In Exp. 1, mice were given PG-TXL or TXL at an equivalent TXL dose of 60 mg/kg as a single i.v. injection. In Exp. 2, the dose of PG-TXL was 80 mg equivalent TXL/kg. When taxane and irradiation were combined, local irradiation was delivered 24 h before PG-TXL treatment. The groups consisted of six mice each.

[c] Absolute growth delay was defined as the time in days for tumors in treated groups to grow from 8 to 12 mm minus the time in days for tumors in the saline-treated control group to grow to the same size.

[d] Normalized growth delay was defined as the time in days for tumors to grow from 8 to 12 mm in mice treated with the combination of radiation and drug minus the time in days for tumors to grow to the same size in mice treated with the same dose of radiation alone.

[e] Enhancement factors were obtained by dividing normalized tumor growth delay by the absolute growth delay in mice treated with drug only. CI, confidence interval.

[f] Analysis was based on five mice; one of six mice had total tumor control at 180 days after the treatment.

[g] Analysis was based on four mice; two of six mice had total tumor control at 180 days after the treatment.

and 48 h after irradiation with 10 and 15 Gy using ELISA. The mean serum VEGF concentrations increased from a pretreatment level of 60.4 ± 13.8 pg/ml to a posttreatment level of 119.5 ± 37.9 pg/ml at 48 h after irradiation ($P = 0.005$).

Irradiation Arrests Tumor Cells in G_1 Phase of the Cell Cycle. To determine whether irradiation increased cellular sensitivity to PG-TXL, we analyzed the cell cycle distribution of OCa-1 tumor cells 24 h after irradiation with 15 Gy. Flow cytometry revealed that the percentage of cells in the G_1 phase was 64% in control samples and 77% 24 h after irradiation ($P = 0.014$). This resulted in a decrease in the percentage of cells in S phase from 26% in control samples to 13% in irradiated samples ($P = 0.0105$). Irradiation did not cause measurable changes in the population of G_2-M-phase cells (9.4% and 9.5% for control and irradiated samples, respectively).

DISCUSSION

In the treatment of many solid tumors, combining chemotherapy and radiotherapy has produced significantly improved response and survival rates compared to treatment with either modality alone (12, 13). However, local recurrence and systemic relapse remain the major obstacles to cure. There is still a compelling need to establish the most effective sequence of therapies, the best chemotherapeutic agents, and the most appropriate way to deliver these agents. Both *in vitro* and *in vivo* studies have demonstrated that TXL given before radiation can strongly enhance tumor radioresponse (14–16). In animal studies, the enhancement factors range from 1.2 to more than 2.0, depending on the tumor type, drug concentration, and dose scheduling (10, 16). However, to our knowledge, no previous *in vivo* study has examined the ability of radiation to increase the therapeutic effectiveness of TXL. In fact, *in vitro* experiments in which TXL was added posirradiation demonstrated a subadditive effect of the two modalities (17–19).

The present study attempts to examine whether radiation can be used to enhance tumor response to macromolecular chemotherapy *in vivo*. We found that treatment with radiation followed 24 h later by PG-TXL caused a significantly longer tumor growth delay than did treatment with PG-TXL alone, implying that radiation increased tumor responsiveness to PG-TXL (Fig. 1). Furthermore, the enhanced tumor growth delay was much greater when radiation was combined with PG-TXL than when it was combined with TXL, suggesting that conjugation to poly(L-glutamic acid) is necessary for improved antitumor efficacy (Table 1). The enhanced chemoresponsiveness was evident at a radiation dose as low as 5 Gy, and the enhancement increased as the radiation dose increased (Table 1). The enhancement factors of 2.35 and 2.87 for chemoresponse to PG-TXL obtained at radiation doses of 10 and 15 Gy could be underestimated because some mice in the two treatment groups had tumor regression and were not used in data calculation. The dose of PG-TXL used in the current study, 60–80 mg equivalent TXL/kg, was 2.0–2.67-fold below the maximum tolerated dose of 160 mg equivalent TXL/kg (5). No apparent toxic effects were observed at the doses of PG-TXL and radiation used. Thus, a broad therapeutic window may be achievable with this novel treatment strategy. An important next step is to determine whether the antitumor activity of combined therapy

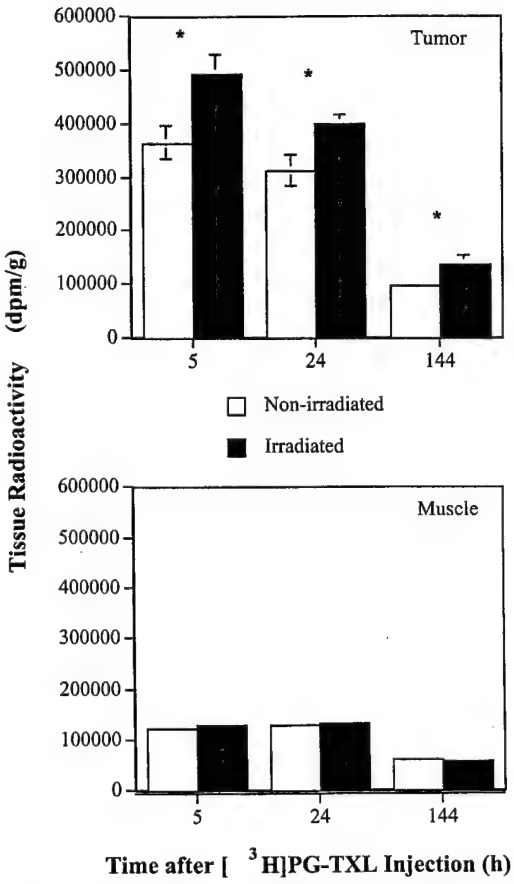

Fig. 2 Radioactivity in OCa-1 tumor and contralateral muscle after i.v. injection of [^{3}H]PG-TXL into nonirradiated mice or mice treated with local irradiation at a dose of 15 Gy. Data are presented as the means from four mice/time point; *bars*, SE; *asterisks*, significant difference between groups.

could be maximized using fractionated clinically relevant radiation doses (*i.e.*, 5 Gy/dose for five doses) and multiple injections of PG-TXL.

Enhancement of chemoresponse to PG-TXL by radiation can be explained in part on the basis of increased tumor uptake of PG-TXL. Tumor accumulation of PG-TXL at 24 h after irradiation increased 28–38%, and PG-TXL was retained in the tumor for a prolonged period of time (Fig. 2). The increased tumor uptake of PG-TXL most likely resulted from increased vascular permeability induced by local tumor irradiation. As measured by the Evans Blue dye extravasation assay, the vascular permeability increased 26% at 24 h after irradiation. Consistent with the role of VEGF as a potent mediator of tumor angiogenesis and vascular permeability, we observed elevated serum VEGF concentrations after irradiation at doses ranging from 10–15 Gy, possibly resulting from tumor response to radiation stress. Interestingly, Gorski *et al.* (20) reported recently that exposure to ionization radiation induces VEGF expression in Lewis lung carcinomas both *in vitro* and *in vivo* and in human tumor cell lines and that blocking the effect of VEGF enhances the antitumor effects of irradiation.

Our results support the view that radiation enhances the EPR effect of macromolecules, causing an increase in tumor vascular permeability and allowing more PG-TXL to enter the tumor. This results in a high and prolonged concentration of PG-TXL in surviving tumor cells. The amount of TXL per viable tumor cell was higher as the radiation dose was increased because fewer viable cells remained in the tumor after higher radiation doses. This could explain the observed increase in enhancement factor values as the radiation dose was increased (Table 1). In a previous study, we demonstrated that increased tumor uptake of PG-TXL and sustained release of TXL from PG-TXL in the tumor were responsible for the enhanced antitumor activity of PG-TXL compared with TXL formulated in a cremophor/alcohol vehicle.[4] Both this previous study and the current study reached the same conclusion: that the EPR effect is an important mechanism through which use of macromolecular chemotherapeutic agents enhances antitumor efficacy and improves the therapeutic index.

Radiation treatment of OCa-1 tumor in the present study also caused cell cycle redistribution and resulted in a significant increase in the percentage of G_1 cells at the time of PG-TXL administration (24 h after irradiation). Talwar and Redpath (18) examined the interaction of radiation and TXL in HeLa cells using a protocol in which cells were exposed to 7 Gy and then treated with TXL after a variable interval (0–24 h). The authors analyzed their data in terms of redistribution of cells in the phases of the cell cycle and concluded that subadditive, additive, and supra-additive interactions can be observed, depending on the treatment schedule and the corresponding cell cycle distribution. Maximum cell killing occurred when the percentage of cells in G_1 phase was at a minimum at the time of TXL treatment (18). According to the findings of Talwar and Redpath (18), the schedule used in the present study should have resulted in reduced efficacy of PG-TXL and TXL. However, our data showed potentiation of the efficacy of TXL (enhancement factor, 1.50) and a remarkably strong potentiation of the efficacy of PG-TXL (enhancement factor, 4.44). Therefore, the radiation-induced enhancement of OCa-1 response to PG-TXL and TXL observed in the *in vivo* setting cannot be explained on the basis of radiation-induced cell cycle redistribution. Obviously, additional detailed studies are needed to clarify the contradictory results between *in vitro* and *in vivo* findings.

In summary, our data suggest that radiation may be used to modulate tumor vascular permeability and the EPR effect of macromolecular chemotherapeutic agents. The treatment strategy that combines radiotherapy and macromolecular chemotherapy as demonstrated in the present study may have important clinical implications in terms of treatment scheduling and optimization of the therapeutic ratio. Additional studies are needed to determine whether enhancement of the EPR effect by radiation also occurs in other tumors and with other macromolecular chemotherapeutic agents.

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Potentiation of radioresponse by polymer–drug conjugates

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Abstract

Although combined chemotherapy and radiotherapy has produced significantly improved response and survival rates among cancer patients, there is still a compelling need to establish the most effective way to deliver these agents. We hypothesize that the radiosensitizing effect of a chemotherapeutic agent can be further enhanced if the drug is delivered at an optimal concentration and is maintained in the tumor for a prolonged period. Using a water-soluble poly(L-glutamic acid)-conjugated paclitaxel (PG–TXL) as a model compound, we investigated whether paclitaxel delivered by means of polymeric carrier could increase the tumor's response to radiation. Mice bearing 8-mm syngeneic ovarian carcinoma OCa-1 tumors implanted intramuscularly were treated with i.v. injected PG–TXL alone or in combination with single doses of local radiation. The enhancement factors at 24 h interval, as measured by incremental tumor growth delay compared with radiation alone, ranged from 2.48 to 4.28. The values varied as a function of radiation dose. The enhancement of radioresponse is also a function of time interval between injection of PG–TXL and tumor irradiation. The enhancement factor increased with decreasing interval, suggesting that radiation may in turn mediate the sensitivity of tumor toward PG–TXL. Thus, the mechanism of PG–TXL's radiopotentiation activity is probably multifactorial. Remarkably, while combined radiation and TXL produced additive or even sub-additive interaction when radiation preceded TXL injection, combined radiation and PG–TXL produced synergistic interaction in a mammary MCa-4 tumor model. Radiation significantly increased tumor uptake of PG–TXL, suggesting a potential role of radiation-modulated antitumor activity of polymeric drugs. Our data support a treatment strategy combining radiation and polymeric chemotherapy that may have important clinical implications in terms of scheduling and optimization of the therapeutic ratio.

Keywords: Radiosensitization; Combination therapy; Paclitaxel; Polymer

1. Introduction

In the treatment of many solid tumors, combining chemotherapy and radiotherapy has produced significantly improved response and survival rates. This is because radiotherapy is most effective in controlling local-regional disease whereas chemotherapy can attack distant metastases. Furthermore, many chemotherapeutic agents are able to increase the sensitivity of tumor to radiation, therefore potentiate tumor response to radiation caused damage. In spite of extensive efforts in identifying the best chemotherapeutic agents as potential radiosensitizer and in establishing the most effective schedule in combined therapy, few studies have investigated how the way

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the drug is delivered might have impacted on the outcome of radiochemotherapy.

Our work was conducted to test the following two hypotheses. (1) A sustained release drug delivery system that localizes the drug to the tumor and releases it slowly over time could enhance the responsiveness of solid tumor to radiotherapy. (2) Radiation may potentiate tumor response to polymeric drug conjugates by increasing tumor vascular permeability and thus increasing their uptake in solid tumor. To test these hypotheses, we used poly(L-glutamic acid)-paclitaxel (PG–TXL) as a model polymeric drug conjugate. Both in vitro and in vivo studies have shown that paclitaxel (TXL) can strongly enhance tumor cell's sensitivity to radiation. However, considerable variations in radiosensitization, including a supra-additive, additive, and a sub-additive effect, have been observed [1–5]. PG–TXL is a water-soluble poly(L-glutamic acid)-conjugated TXL that has shown significant antitumor and antimetastatic activity in a variety of animal tumor models [6,7]. Biodistribution studies using PG-[^{3}H]TXL and [^{3}H]TXL revealed that tumor uptake of TXL after injection of PG-[^{3}H]TXL is five-fold higher than after the injection of [^{3}H]TXL when both drugs were injected at the same equivalent TXL dose, and that free TXL was released from PG–TXL within the tumor over a prolonged period of time [8]. Thus, PG–TXL is a suitable model compound to test our hypotheses.

2. Materials and methods

TXL (Hande Tech, Houston, TX, USA) was dissolved in Cremophor/alcohol (1:1, v/v) at a concentration of 30 mg/ml. The stock solution was diluted with saline (1:4, v/v) immediately before injection. PG–TXL (molecular weight, 36,000–49,000; TXL loading, 12.8–26.8%) was synthesized in our laboratory according to previously described procedures [6]. The conjugate was dissolved in saline at an equivalent TXL concentration of 8 mg/ml and filtered through a 0.22-μm sterile filter before injection.

Solid murine ovarian OCa-1 tumors or mammary MCa-4 tumors were generated in the muscles of the right thigh of female C3Hf/Kam mice by inoculating 5×10^5 viable tumor cells in suspension in phosphate buffered saline. The antitumor effects of radiation alone, taxanes alone, and combined therapy with a single i.v. injection of PG–TXL or TXL given prior to or after tumor irradiation were determined in terms of their ability to delay tumor growth. When tumors had grown to 8 mm in average diameter, mice were randomly allocated into groups of six. Single doses of 5, 10, or 15 Gy were delivered to the tumor with a ^{137}Cs source at a dose rate of 6.25 Gy per minute. PG–TXL at an equivalent TXL dose of 80 mg/kg was injected i.v. via the tail vein. Tumor growth was determined daily by measuring three orthogonal tumor diameters. The mice were sacrificed when tumors reached 14 mm in diameter.

[^{3}H]PG–TXL with tritium labeled to poly(L-glutamic acid) was used to evaluate the effect of radiation on tumor uptake of polymeric drug conjugate. Mice with OCa-1 tumors of 8 mm in average diameter were divided into two groups of four mice each. Mice in group 1 were injected i.v. with [^{3}H]PG–TXL at an equivalent TXL dose of 20 mg/kg (8 μCi). Mice in group 2 were treated with 15 Gy local tumor irradiation, followed by an i.v. injection of the same dose of [^{3}H]PG–TXL 24 h later. Animals were sacrificed at 5, 24, or 144 h after injection of [^{3}H]PG–TXL. The radioactivities in muscle and tumor tissues were counted and the data were expressed as dpm/g.

The flow cytometry analysis of cell cycle distribution was performed using OCa-1 tumors collected 4, 24, 48, and 72 h after i.v. injection of PG–TXL at an equivalent TXL dose of 80 mg/kg. Control mice were not treated. Cells were released from tumor tissue by cutting the tumor in half and scraping the surface. The cell suspension was then stained with fluorochrome buffer (0.1% sodium citrate, 0.3% Triton X-100, 20 μg/ml RNAse, 50 μg/ml propidium iodide) in the dark at 4°C overnight. The cellular content was analyzed using a Coulter SL flow cytometer (Coulter, Miami, FL, USA) after the cells were filtered through a 37-μm nylon mesh (Spectrum).

3. Results and discussion

When used alone, PG–TXL or radiation caused

significant tumor growth delay. However, when PG–TXL was combined with radiation with 24 h interval, tumor growth delay in days was markedly increased [10] (Fig. 1). The normalized growth delay, defined as the time in days for tumors to grow from 8 to 12 mm in mice treated with the combination of PG–TXL and radiation minus the time in days for tumors to grow to the same size in mice treated with the same dose of PG–TXL alone, increased by 16, 28, and 67 days. This yielded enhancement factors (obtained by dividing normalized growth delay by the growth delay in mice treated with radiation alone) of 2.79, 2.48, and 4.28 at radiation doses of 5, 10, and 15 Gy, respectively. These values were substantially greater than those obtained with TXL in the same tumor model under similar experimental conditions. For example, an enhancement factor of 1.66 was obtained when TXL was given i.v. at a dose of 40 mg/kg in combination with 14 Gy local tumor irradiation with 24 h interval [9]. Thus, by simply conjugating TXL to a water-soluble polymer, the radiosensitizing effect of the parent drug was further improved by a factor of 1.5 to 2.5. These results suggest that the sustained release of chemotherapeutic agents realized by polymeric drug conjugates enhances radioresponse of tumor during the critical period of radiotherapy.

TXL's radiosensitizing effect has previously been attributed to its ability to arrest tumor cells in the G2/M phase of the cell cycle because G2/M cells are most sensitive to ionizing radiation [1]. To study whether the enhanced radiosensitizing activity of PG–TXL is a result of cell cycle redistribution, we analyzed OCa-1 cells at different times after PG–TXL treatment by flow cytometry. Treatment with PG–TXL at an equivalent TXL dose of 80 mg/kg increased the percentage of G2/M cells from 14.6 to 21.4% ($P = 0.0004$) 48 h after drug injection. By 72 h, the percentage of cells in G2/M phase declined to the preinjection level. Thus, the enhanced radioresponse of OCa-1 tumor within 48 h after PG–TXL treatment may be attributed in part to the arrest of tumor cells in the G2/M phase.

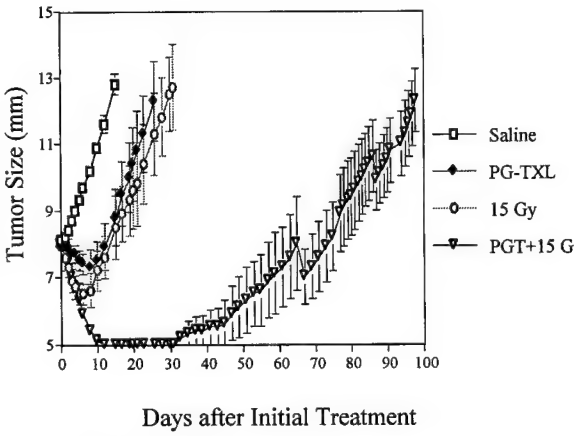

Fig. 1. Enhancement of ovarian OCa-1 tumor radioresponse by PG–TXL. (●) Tumors were treated when the average diameter reached 8 mm. PG–TXL was given as a single i.v. injection at 80 mg eq. TXL/kg 24 h *before* IR. The EF was 4.28. From Ref. [10] with permission.

Milas et al. has previously reported the effect of the interval between TXL injection and radiation delivery in OCa-1 tumor [9]. They found that the enhancement of tumor radioresponse increased as the interval increased up to 48 h, then decreased at later times. This is attributed to the time needed for TXL to induce tumor apoptosis and tumor reoxygenation. If modulation of cell cycle distribution and subsequent apoptosis played a major role in PG–TXL's radiopotentiating effect, one would expect that a peak enhancement of radioresponse would be reached at a time interval equal or greater than 48 h because of the extra time needed for TXL to release from PG–TXL. To investigate whether this is the case, the effect of the interval between the injection of PG–TXL and delivery of radiation on the radiopotentiating effect of PG–TXL was studied and the result is plotted in Fig. 2. PG–TXL not only produced a stronger radiopotentiating effect than TXL at each time point within 48 h, the kinetics of the radiopotentiating effect of PG–TXL is also different from that of TXL. For TXL, a peak enhancement factor of 1.96 was reached when the time interval was 48 h [9]. For PG–TXL, the enhancement factor increased when the interval between treatments decreased. An enhancement factor of 3.69 was obtained when PG–TXL was given at an equivalent TXL dose of 80 mg/kg and the tumor was irradiated 4 h later at a dose of 10 Gy. Obviously, factors other than TXL-induced mitotic arrest and apoptosis may also play a role in PG–TXL's strong radiopotentiating activity.

The observation that a shorter interval between

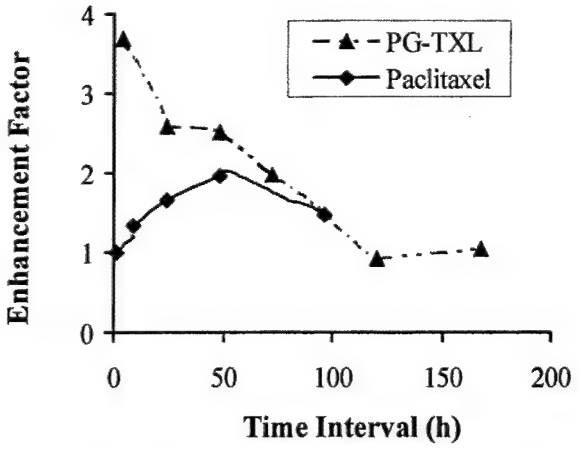

Fig. 2. Enhancement factor as a function of interval. Data for TXL were taken from Ref. [1]. TXL, 40 mg/kg; IR, 14 Gy; PG–TXL, 80 mg eq./kg; IR, 10 Gy. Mice with OCa-1 tumor were treated first with Taxanes, followed by IR. From Ref. [10] with permission.

injection of PG–TXL and delivery of radiation resulted in a stronger synergistic interaction is intriguing. It suggests that in addition to PG–TXL's radiosensitization effect, radiation may in turn potentiate PG–TXL's antitumor activity. In fact, it has been shown previously that radiation increases the vascular permeability of solid tumors [11,12]. These findings prompted us to hypothesize that enhancement of vascular permeability by radiation may cause enhanced tumor uptake of polymeric drug conjugates, leading to improved antitumor efficacy. The same OCa-1 tumor model was used to test our hypothesis using the same experimental conditions except that the tumors were irradiated 24 h prior to i.v. injection of either PG–TXL or TXL. As shown in Fig. 3, combined radiation (15 Gy) and PG–TXL (60 mg equivalent TXL/kg) caused remarkably longer tumor growth delay than combined radiation (15 Gy) and TXL (60 mg/kg) therapy [13] (Fig. 3). The enhancement factors obtained were 4.44 and 1.50 for PG–TXL and TXL, respectively.

To determine whether prior irradiation indeed affects tumor uptake of PG–TXL, [^{3}H]PG–TXL was injected into mice with OCa-1 tumors 24 h after irradiation with 15 Gy. Fig. 4 shows the uptake of [^{3}H]PG–TXL in irradiated and non-irradiated tumors determined at 5, 24, and 144 h after injection of [^{3}H]PG–TXL. The uptake of [^{3}H]PG–TXL in ir-

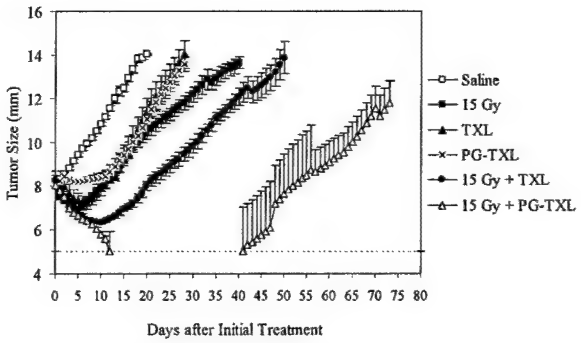

Fig. 3. Effect of radiation and PG–TXL on the growth of ovarian OCa-1 tumors in mice. Tumors were treated when the average diameter reached 8 mm. PG–TXL or TXL was given as a single i.v. injection at 60 mg eq. TXL/kg 24 h *after* irradiation. From Ref. [13] with permission.

radiated tumors was 35, 28, and 38% higher than that in non-irradiated tumors at 5, 24, and 144 h after [^{3}H]PG–TXL injection, respectively. In contrast, no difference was found in the uptake of [^{3}H]PG–TXL in muscle tissues taken from the contralateral, unexposed thigh in irradiated and non-irradiated mice

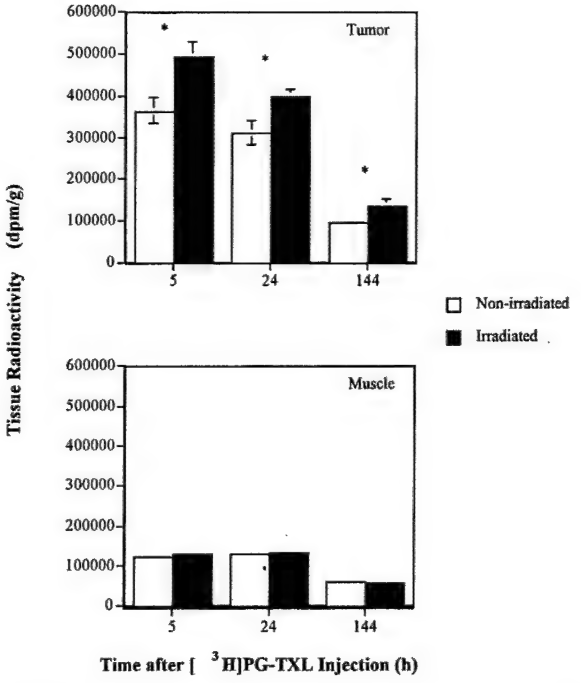

Fig. 4. Tumor irradiation enhances the tumor-specific distribution of PG–TXL. From Ref. [13] with permission.

Table 1
Radiation-induced enhancement of mammary MCa-4 tumor response to PG–TXL

Treatment	Time interval between IR and taxane (h)	Absolute growth delay (days)[a]	Normalized growth delay (days)[b]	Enhancement factor[c]
15 Gy		5.5		
PG–TXL		5		
TXL		7		
15 Gy+PG–TXL	1	16	10.5	2.1
15 Gy+PG–TXL	4	15.5	10.0	2.0
15 Gy+PG–TXL	24	14.5	9.0	1.8
15 Gy+PG–TXL	48	11.5	6.0	1.2
15 Gy+TXL	1	11.5	6.0	0.86
15 Gy+TXL	4	13	7.5	1.1
15 Gy+TXL	24	11.5	6.0	0.86

[a] Absolute growth delay was defined as the time in days for tumors in treated groups to grow from 8 to 12 mm minus the time in days for tumors in the saline-treated control group to grow to the same size.

[b] Normalized growth delay was defined as the time in days for tumors to grow from 8 to 12 mm in mice treated with the combination of radiation and drug minus the time in days for tumors to grow to the same size in mice treated with radiation alone.

[c] Enhancement factors were obtained by dividing normalized tumor growth delay by the absolute growth delay in mice treated with drug only.

(Fig. 4). Thus, tumor irradiation increased accumulation of PG–TXL in tumors for at least 6 days.

The effect of the time interval between tumor irradiation and injection of PG–TXL was investigated using mammary MCa-4 tumor. Tumors irradiated at 1–24 h prior to injection of PG–TXL resulted in significant tumor growth delay, yielding enhancement factors of about 2.0 (Table 1). Interestingly, combined radiation and TXL yielded enhancement factors of 0.86 to 1.0, indicating that only additive or even sub-additive interactions were produced when radiation preceded TXL treatment. This observation is in agreement with in vitro findings that suggest radiation could antagonize the cytotoxicity of TXL [4,5]. Prior radiation can modify the effect of subsequent TXL treatment by inducing cell cycle delay. It is possible that by using a polymeric conjugate such as PG–TXL, the effect of altered cell cycle caused by radiation is reduced to a minimum because the drug persisted in the tumor for a longer period of time.

4. Conclusion

Polymer–drug conjugates exemplified by PG–TXL provide a stronger radiosensitization effect than the parent drug TXL in a sequence-independent manner. When PG–TXL precedes tumor irradiation, the synergistic interaction between PG–TXL and radiation is caused by several factors including modulation of cell cycle, induction of apoptosis, persistent concentration of the drug in the tumor, and possibly other mechanisms. When radiation proceeds PG–TXL treatment, the synergistic interaction between tumor irradiation and PG–TXL is in part attributable to modulation of the EPR (enhanced permeability and retention effect) of polymers. Combining polymeric drug chemotherapy with radiation may have important clinical implications in terms of scheduling and optimization of the therapeutic ratio.

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BIOLOGY CONTRIBUTION

POLY(L-GLUTAMIC ACID)-PACLITAXEL CONJUGATE IS A POTENT ENHANCER OF TUMOR RADIOCURABILITY

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Purpose: Conjugating drugs with polymeric carriers is one way to improve selective delivery to tumors. Poly (L-glutamic acid)-paclitaxel (PG-TXL) is one such conjugate. Compared with paclitaxel, its uptake, tumor retention, and antitumor efficacy are increased. Initial studies showed that PG-TXL given 24 h before or after radiotherapy enhanced tumor growth delay significantly more than paclitaxel. To determine if PG-TXL-induced enhancement is obtained in a more clinically relevant setting, we investigated PG-TXL effects on tumor cure.

Methods and Materials: Mice bearing 7-mm-diameter ovarian carcinomas were treated with PG-TXL at an equivalent paclitaxel dose of 80 mg/kg, single dose or 5 daily fractions of radiation or both PG-TXL and radiation. Treatment endpoint was TCD_{50} (radiation dose yielding tumor control in 50% of mice). Acute radioresponse of jejunum, skin, and hair was determined for all treatments.

Results: PG-TXL dramatically improved tumor radioresponse, reducing TCD_{50} of single-dose irradiation from 53.9 (52.2–55.5) Gy to 7.5 (4.5–10.7) Gy, an enhancement factor (EF) of 7.2. The drug improved the efficacy of fractionated irradiation even more, reducing the TCD_{50} of 66.6 (62.8–90.4) Gy total fractionated dose to only 7.9 (4.3–11.5) Gy, for an EF of 8.4. PG-TXL did not affect normal tissue radioresponse resulting from either single or fractionated irradiation.

Conclusion: PG-TXL dramatically potentiated tumor radiocurability after single-dose or fractionated irradiation without affecting acute normal tissue injury. To our knowledge, PG-TXL increased the therapeutic ratio of radiotherapy more than that previously reported for other taxanes, thus, PG-TXL has a high potential to improve clinical radiotherapy. © 2003 Elsevier Science Inc.

Poly(L-glutamic acid)-paclitaxel, Radiotherapy, Tumor cure, Therapeutic gain.

INTRODUCTION

The combination of chemotherapy and radiotherapy has become common in the therapy of locally advanced cancer. Recently, concurrent chemoradiotherapy has been emphasized because increasing evidence shows that it improves both local tumor control and survival in patients with any one of a number of common cancers (1–3). However, concurrent chemoradiotherapy is often associated with increased normal tissue toxicities that limit its application. Therefore, new means are being explored to selectively target tumors with chemotherapeutic agents to reduce normal tissue toxicity and maximize antitumor efficacy. For example, capecitabine is an orally available fluoropyrimidine carbamate that is converted to 5-fluorouracil (5-FU) via a sequential triple enzymatic pathway that exploits the higher tumor concentration of the enzyme thymidine phosphosylase (TP) (4). TP activity has been reported to be 3–10 times higher in human tumors than in corresponding normal tissue (5). Thus, 5-FU is delivered preferentially to the tumor. Another approach is the use of hypoxic cell cytotoxins such as tirapazamine. Since for all practical purposes, normal tissues are well oxygenated, hypoxic cell cytotoxins selectively target tumors (6). Furthermore, recent advances in molecular biology have detected many potential targets for augmentation of radio- or chemotherapeutic response such as epidermal growth factor receptor, cyclooxygenase-2 enzyme, the *ras* gene, and angiogenic molecules (7).

Conjugating chemotherapeutic drug with polymer carriers is yet another way to improve selective drug delivery to tumors. The approach investigated here consists of the use

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of poly(L-glutamic acid)-paclitaxel (PG-TXL), a water-soluble conjugate that allows higher concentrations of TXL to be delivered selectively to tumors. Unconjugated paclitaxel is water insoluble. It can be dissolved in cremophor plus ethanol, but that formulation is associated with toxic reactions such as hypersensitivity reactions, hypotension, and cardiac arrhythmia (8). Biodistribution studies of the conjugate in tumor-bearing mice showed increased uptake of PG-TXL by tumors and longer tumor retention compared with that of unconjugated paclitaxel (9, 10). The enhanced uptake and prolonged release of PG-TXL by tumors is thought to be due to the enhanced permeability and retention effect (EPR) of macromolecular drugs in solid tumors (11–13). The abnormal vasculature in tumors is porous to macromolecules, but high concentrations of drug can build up in tumors because of inadequate lymphatic drainage, whereas polymer-drug conjugates are confined to the bloodstream in normal tissue (11).

Preclinical studies in mice and rats showed that PG-TXL can be given in higher doses than unconjugated paclitaxel (without causing toxicity) and that PG-TXL exerts much stronger antitumor efficacy than paclitaxel against both syngeneic tumors and human tumor xenografts (14, 15). The superior antitumor efficacy of PG-TXL is attributed to higher tumor uptake and longer retention of the drug (10). A logical consequence of these findings has been for investigators to explore the efficacy of PG-TXL in combination with radiation, particularly given recent observations that taxanes, both docetaxel and TXL, are potent enhancers of *in vitro* tumor cell radiosensitivity and *in vivo* tumor radioresponse (16). Taxanes enhance tumor radioresponse by a number of mechanisms, primarily by arresting tumor cells in the G_2/M radiosensitive phases of the cell cycle (17, 18) and by reoxygenation of hypoxic tumor cells (19), which are 2.5 to 3 times more resistant to radiation than well-oxygenated cells (20). The degree of enhancement of both *in vitro* cell and *in vivo* tumor radioresponse generally did not exceed a factor of 2 (16). Compared with tumors, normal tissue radioresponse after single-dose or fractionated radiation was less affected by taxanes, showing that these drugs are able to significantly increase the therapeutic ratio of radiotherapy (16, 21).

In our initial studies on the combination of PG-TXL and radiation, we observed that PG-TXL given 24 h before or 24 h after tumor irradiation of the murine ovarian carcinoma OCA-I, increased the efficacy of tumor radiation by a factor of more than 4 (19, 20). PG-TXL containing 60 mg/kg paclitaxel, given 24 h after tumor irradiation, enhanced tumor radioresponse to a single dose of 15 Gy by a factor of 4.44, whereas 60 mg/kg unconjugated paclitaxel given under the same treatment conditions enhanced it by a factor of only 1.5 (22). The enhancement factor generated from the radiation dose–response curves, which was defined as the ratio of the slopes of the combined modality treatment to the slope of the radiation-only treatment, was 5.13 when PG-TXL was given 24 h before irradiation (23). These initial observations were made using single-dose irradiation and tumor growth delay as the treatment endpoint. The experiments described in this report were designed to investigate whether this dramatic enhancement of tumor radioresponse produced by PG-TXL is maintained in more relevant clinical settings, i.e., when PG-TXL is combined with fractionated irradiation given daily and when the treatment endpoint is tumor cure. In addition, we investigated whether PG-TXL modifies normal tissue radioresponse to fractionated irradiation to determine its potential for increasing therapeutic ratio of radiotherapy.

METHODS AND MATERIALS

Mice

Three- to five-month-old C3Hf/Kam mice bred in our specific-pathogen-free facility were used. They were housed 4–5 per cage, exposed to 12-h light-dark cycles, and given free access to sterilized pelleted food (Prolab Animal Diet, Agway Inc., Syracuse, NY) and sterilized water. Mice were maintained in an American Association for Laboratory Animal Care accredited facility and in accordance with current regulations of the U.S. Departments of Agriculture and Health and Human Services. The experimental protocol was approved by, and in accordance with, institutional guidelines established by the Institutional Animal Care and Use Committee.

PG-TXL

PG-TXL (CT-2103), molecular weight of 36,000–49,000 containing paclitaxel loading 37%, was supplied by Cell Therapeutics Inc., (Seattle, WA). The conjugate was dissolved in disodium phosphate buffer to an equivalent paclitaxel concentration of 8 mg/mL. The tail vein of each mouse was injected with a paclitaxel equivalent dose of 80 mg/kg body weight.

Tumor

Studies were performed using a transplantable syngeneic ovarian adenocarcinoma (OCA-I) in its seventh isotransplant generation. This tumor originally arose spontaneously and has since been stored in liquid nitrogen. Solitary tumors were established in the muscle of the right leg of experimental mice by injection of a single-cell suspension of 5×10^5 viable tumor cells. The suspensions were prepared by mechanical disruption and enzymatic digestion of parent tumors, as previously described (24).

Tumor irradiation procedure and response measurement

When they grew to 7 mm in mean diameter, tumors were locally irradiated using a small animal irradiator consisting of parallel opposed ^{137}Cs sources at a dose rate of 5.9 Gy/min. During irradiation the mice were not anesthetized, but they were immobilized in a jig with the tumor centered in the 3-cm-diameter circular treatment field. Radiation doses ranged from 1 to 16 Gy given daily for 5 days (5 to 80 Gy total dose). Tumor-bearing mice were treated with 80 mg/kg PG-TXL 24 h before the first radiation exposure.

Mice were checked for the presence of tumor at the irradiated site at 2- to 8-day intervals for up to 120 days. Tumor size during regression and regrowth was determined by measurement of three orthogonal tumor diameters with Vernier calipers. Tumors were considered postradiation recurrences when tumors that had regressed regrew to 7 mm in diameter. The mice were killed when their tumors grew to approximately 14 mm. TCD_{50} (radiation dose yielding 50% tumor control) values were computed by the logit method of analysis (25). To assess the therapeutic efficacy of PG-TXL only, a group of tumor-bearing mice that received no treatment and a group of mice that received 80 mg/kg PG-TXL were also included.

Normal tissue irradiation procedure and response measurement

Jejunum. The microcolony assay introduced by Withers and Elkind (26) was used to determine the survival of crypt epithelial cells in the jejunum of mice exposed to radiation. Mice were exposed to whole-body irradiation (WBI) with single doses of 300-kV X-rays ranging from 9.75 to 13.25 Gy given once or daily doses of 4.0 to 6.4 Gy for total doses of 24.5–32 Gy for 5 consecutive days at a dose rate of 1.84 Gy/min. The mice were treated i.v. with 80 mg/kg PG-TXL 24 h before the first radiation exposure and were killed 3.5 days after WBI was completed. The jejunum was prepared for histologic examination, and the number of regenerating crypts in the jejunal cross-section was counted. To construct radiation survival curves, the number of regenerating crypts was converted to the number of surviving cells by applying a Poisson correction for crypts regenerating from more than one stem cell. Lines were fitted to data points by least-squares regression analysis.

Skin. The effect of PG-TXL on radiation injury to the skin was determined from mice used in the TCD_{50} assays that had no recurrent tumors present. The skin of the irradiated legs was assessed both for skin desquamation and hair loss. Skin desquamation was scored daily on the ventral surface of the leg using a scale of 0–3.5, representing the degree of dry and moist desquamation (27). Reactions were scored starting 16 days after the first radiation dose and continuing until the moist reaction began to heal (up to 34 days after treatment). The peak reaction during the scoring period was used as the response value for each mouse. Radiation-induced hair loss (epilation) on the dorsal surface of the irradiated leg was examined 28–31 days after single-dose irradiation and 30–34 days after the start of fractionated treatment. An arbitrary semiquantitative scale of 0–4 was used to determine the percentage of hair loss, where 0 represents no change and 4 represents complete epilation (28).

RESULTS

Tumor radioresponse

To determine whether PG-TXL increases tumor radiocurability, TCD_{50} experiments were performed in which PG-TXL was combined with single or fractionated irradiation. The mice bearing 7-mm OCA-I tumors were treated i.v. with PG-TXL, at a paclitaxel equivalent dose of 80 mg/kg, or vehicle, and 24 h later their tumors were exposed to a range of single doses of radiation or to a 5-dose fractionated regimen. Single irradiation doses ranged from 5 to 60 Gy. Each fractional dose ranged between 1 and 16 Gy so that the total dose delivered to the tumor was 5 to 80 Gy. Tumor regression and regrowth was followed at 2- to 8-day intervals for up to 120 days from the end of radiotherapy, at which time tumor cure was assessed. The radiation-dose–response curves of all four treatment groups are plotted in Fig. 1. They show that the percentage of tumors cured increased as the total radiation dose increased. The TCD_{50} value in mice treated with vehicle and single-dose radiation was 53.9 (52.2–55.5) Gy (the numbers in parentheses are 95% confidence intervals). This TCD_{50} value increased to 66.6 (62.8–70.4) Gy when daily fractionated irradiation was used. The TCD_{50} increased by a factor of 1.24 ($p < 0.001$). Treatment of mice with PG-TXL dramatically increased tumor radiocurability by single or fractionated irradiation, as shown by a shift in position of the corresponding radiation-dose–response curves to the lower doses of radiation. PG-TXL reduced the TCD_{50} value after single-dose irradiation to 7.5 (4.3–10.7) Gy, a dose reduction factor of 7.2 ($p < 0.001$), and reduced the TCD_{50} value after fractionated irradiation to 7.9 (4.3–11.5) Gy, a dose reduction factor of 8.4 ($p < 0.001$).

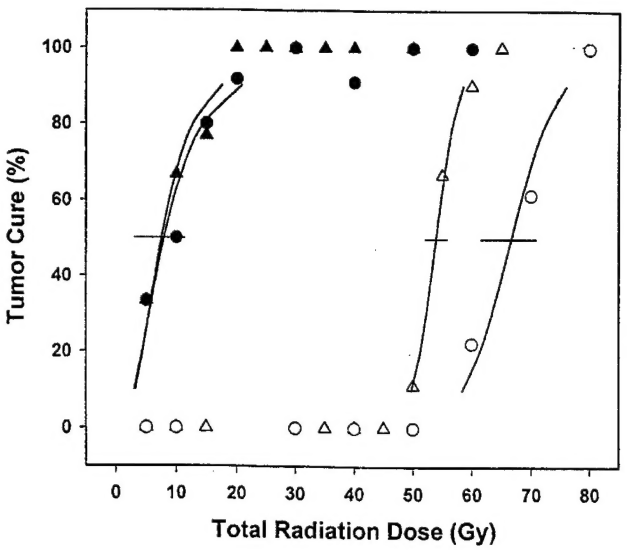

Fig. 1. Effect of PG-TXL on radiocurability of OCA-I tumor after single or fractionated irradiation. Mice bearing 7-mm tumors in the right hind leg were given i.v. 80 mg/kg PG-TXL and/or local tumor irradiation with graded doses of γ-rays delivered as a single dose or as daily fractions for 5 consecutive days. When the two agents were combined, PG-TXL was given 24 h before the start of irradiation. Radiation-dose–response curves were generated for local tumor control at 120 days after treatment with single-dose irradiation alone (Δ), fractionated irradiation ([circo]), PG-TXL plus single-dose irradiation (▲), or PG-TXL plus fractionated irradiation (●). Error bars are 95% confidence intervals on the TCD_{50}.

Table 1. Effect of PG-TXL on radioresponse of OCA-I tumor measured by tumor growth delay

Treatment*	Cured/Total	Tumor growth delay			
		Time in days to grow from 7 to 12 mm	Absolute† growth delay	Normalized‡ growth delay	EF‖
No treatment	0/5	11.6 (10.6–12.6)§			
PG-TXL	0/5	35.5 (27.1–43.9)	23.9 (15.5–32.3)		
5 Gy single-dose radiation	0/5	17.7 (15.9–19.5)	6.2 (4.4–8.0)		
PG-TXL plus 5 Gy single-dose radiation	2/6	60.4 (48.7–72.1)	48.8 (37.1–60.5)	24.8 (21.7–27.9)	4.0
5 Gy fractionated radiation	0/5	17.9 (16.3–19.5)	6.3 (4.7–7.9)		
PG-TXL plus 5 Gy fractionated radiation	2/6	70.8 (58.3–83.3)	59.2 (46.7–71.7)	35.3 (31.2–39.4)	5.6

* Mice bearing 7-mm tumors in the right hind leg were given i.v. 80 mg/kg PG-TXL or 5 Gy local tumor irradiation delivered as a single dose or as 1 Gy daily for 5 consecutive days. When the two agents were combined, PG-TXL was given 24 h before the start of irradiation. Groups consisted of 5 to 6 mice each. The mice whose tumors were cured were excluded from tumor growth delay calculations.

† Absolute tumor growth delay caused by radiation, PG-TXL, or both agents is defined as the time in days tumors required to reach 12 mm from the time of treatment initiation minus the time in days untreated tumors required to grow from 7 to 12 mm.

‡ Normalized tumor growth delay is defined as the time in days for tumors to reach 12 mm in mice treated by the combination of PG-TXL and radiation minus the time in days to reach 12 mm in mice treated by PG-TXL only.

§ Mean and in parentheses 95% confidence intervals.

‖ Enhancement factors: obtained by dividing normalized tumor growth delay in mice treated by PG-TXL plus radiation by the absolute growth delay in mice treated with radiation only.

The observed reductions in TCD_{50} values could have resulted from either the direct cytotoxic action of PG-TXL or the drug's radiosensitizing effects on tumor cells, or both. Because the TCD_{50} assay cannot discriminate between these two effects of PG-TXL, we examined the effect of the drug on radiation-induced tumor growth delay. Treatment of mice bearing 7-mm tumors with PG-TXL, given i.v. at a paclitaxel-equivalent dose of 80 mg/kg, delayed tumor growth more than did 5 Gy of radiation given either as a single dose or as 1-Gy fractions daily for 5 days (Table 1). When PG-TXL was combined with either single or fractionated irradiation, the resulting growth delay was longer than the sum of growth delays produced by individual agents, which indicates that radioenhancement was achieved. To obtain radioenhancement factors produced by PG-TXL, normalized growth delays (see the legend of Table 1) were determined in the combined treatment groups and then divided by the absolute growth delays produced by radiation only. PG-TXL enhanced growth delay after single-dose irradiation by a factor of 4, and that after fractionated irradiation by a factor of 5.6. Thus, radiosensitizing effects of PG-TXL contributed more to the reduction in TCD_{50} values than the cytotoxic action of PG-TXL.

Normal tissue radioresponse

Figures 2 and 3 show the radioresponses of the jejunum, skin, and hair. The response to single or fractionated irradiation of the jejunum was unaffected by exposure to PG-TXL 24 h before irradiation. Similarly, skin desquamation and hair loss were identical in mice treated with radiation only or pretreated with PG-TXL. Thus, toxicity to these epithelial tissues was not increased by PG-TXL.

DISCUSSION

The results of this study show that PG-TXL strongly enhanced tumor response to radiation without affecting the radioresponse of normal tissues (skin, hair, intestinal epithelium). The enhancement was achieved when the drug was combined with fractionated daily irradiation as measured by a tumor cure treatment endpoint. These experi-

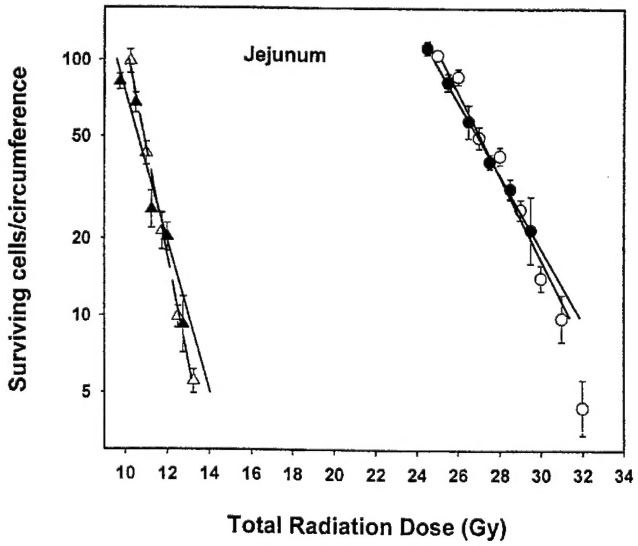

Fig. 2. Effect of PG-TXL on jejunal crypt survival after single or fractionated irradiation. Mice were given PG-TXL at a dose of 80 mg/kg i.v. 24 h before the first dose of radiation. Radiation was given as a single whole-body exposure of 300-kV X-rays or as daily fractions for 5 consecutive days. Radiation dose–response curves were fitted to the data by least-squares regression analysis. Error bars are SEM. Symbols represent single-dose radiation only (Δ), PG-TXL 24 h before single-dose radiation (▲), daily fractionated radiation only (○), and PG-TXL 24 h before the start of fractionated radiation (●).

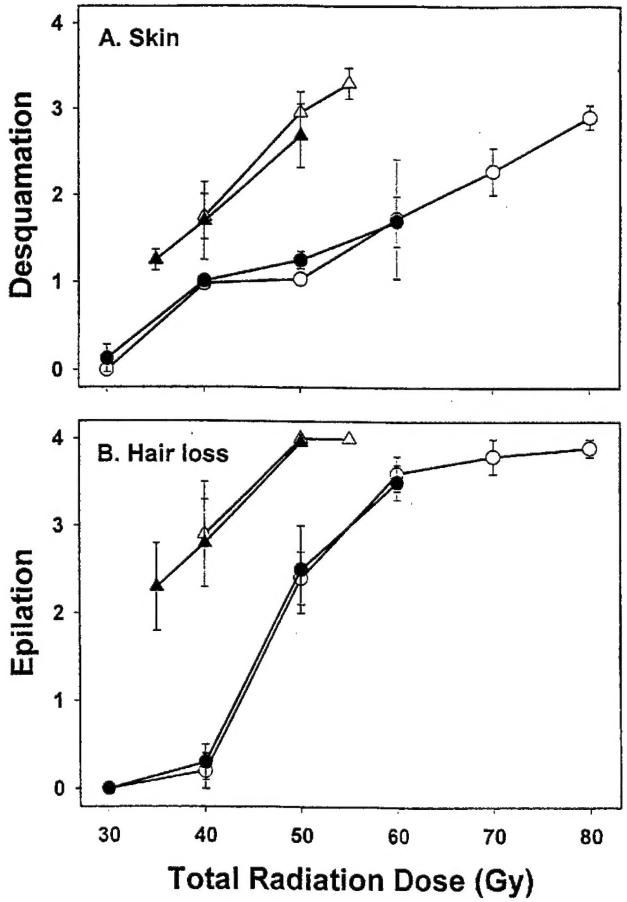

Fig. 3. Effect of PG-TXL on skin desquamation (A) and hair loss (B) after single or fractionated irradiation of the legs of mice. Mice were treated i.v. with PG-TXL at a dose of 80 mg/kg 24 h before the start of irradiation. Radiation was given as a single exposure to 137 Cs gamma rays or as daily fractions for 5 consecutive days. Panel A shows the peak skin reaction during the scoring period of 16–35 days after treatment. Panel B shoes the epilation score (radiation-induced hair loss) on the irradiated leg 28–31 days after single-dose irradiation and 30–34 days after the start of fractionated treatment. Error bars are 95% confidence intervals; open symbols represent mice treated with radiation only, and closed symbols represent mice treated with PG-TXL 24 h before irradiation.

mental settings are highly relevant for clinical application. The effect was better than that when PG-TXL was combined with single-dose irradiation; the EFs being 8.4 after fractionated and 7.2 after single-dose radiation. The magnitude of the enhancement was greater than that produced by other taxanes (16) and, to our knowledge, than that produced by any other chemotherapeutic drug or radiation modifier tested thus far.

The explanation for this dramatic efficacy of PG-TXL must be sought in the EPR phenomenon for macromolecular drugs. Compared with unconjugated TXL, PG-TXL accumulates more in tumors and it is retained there much longer (22). Recently, Li *et al.* (22) demonstrated that PG-TXL is retained in tumors for at least 6 days and that the retained concentration was higher in irradiated than in nonirradiated tumors. Therefore, in the present study PG-TXL was present in OCA-I tumors throughout the fractionated radiotherapy period of 5 days and presumably exerted both its cytotoxic and radiosensitizing effects throughout this time.

The mechanisms by which PG-TXL interacted with radiation were not investigated in the present study; however, they are likely similar to those of other taxanes (reviewed in Ref. 16). As is the case for other taxanes, PG-TXL causes cells to accumulate in the radiosensitive G2 and M phases of the cell cycle (22). This mechanism dominates in tumors that are resistant to taxanes as a single treatment. However, in tumors sensitive to taxanes, such as OCA-I, reoxygenation of radioresistant hypoxic cells is a more dominant mechanism (19, 29, 30). In these tumors the majority of tumor cells that become arrested in mitosis die by apoptosis or necrosis, which occurs within a few days after treatment with taxanes, including PG-TXL (14, 16). This cell loss results in reduction of tumor tissue interstitial fluid pressure (31) and increased tumor pO_2 (19), the consequence of which is a decrease in the proportion of hypoxic cells that are commonly present in solid tumors. Because hypoxic cells are 2.5–3 times more resistant to radiation than well-oxygenated cells, their reoxygenation will increase their radiosensitivity. It is logical to assume that tumor reoxygenation is likely to be greater and of longer duration in tumors treated with PG-TXL than in those treated with paclitaxel because of the EPR effect discussed above. The tumor reoxygenation, which is sustained for a protracted time, would be particularly beneficial for fractionated radiotherapy. This may explain the higher EFs when PG-TXL was combined with fractionated irradiation.

To achieve tumor cure by fractionated radiation, higher doses are required than when single-dose radiation is used, a requirement generally attributed to the repair of sublethal radiation damage after each fraction of radiation and to the tumor cell repopulation that occurs between radiation fractions. In the present study, this requirement for a higher dose in the fractionation arm to achieve tumor cure was likely the result of the repair from radiation damage and not cell repopulation. This conclusion is based on recent findings by Thames *et al.* (32) that tumor cell repopulation does not take place in OCA-I tumors during approximately the first 10 days after the start of fractionated irradiation. Treatment with PG-TXL reduced TCD_{50} values after both fractionated irradiation, which in the present study was delivered in 5 days, and single-dose irradiation to a similar value (below 10 Gy). This better efficacy of PG-TXL in combination with fractionated irradiation could then be attributed, at least partly, to the inhibition of repair from radiation damage. Another explanation is reoxygenation of hypoxic cells as discussed above.

To achieve therapeutic gain in combination with radiotherapy, any chemotherapeutic agent including PG-TXL must increase tumor radioresponse more than radioresponse of critical normal tissues. Our findings show that PG-TXL, given in the same dose and schedules as those used in tumor experiments, had no influence on radiation damage to jejunum or skin inflicted either by single or fractionated irradi-

ation (Figs. 2 and 3). Thus, this treatment dramatically increased the therapeutic ratio of radiotherapy. The lack of sensitization of normal tissues to radiation may be explained by the lack of the EPR phenomenon for macromolecular drugs in normal tissues. Li *et al.* (22) observed that radiolabeled PG-TXL poorly accumulates in normal tissues and that this accumulation is not affected by tissue irradiation.

In summary, PG-TXL combined with fractionated irradiation dramatically increased tumor radiocurability without increasing normal tissue damage by radiation. The increase in tumor cure was in part attributed to independent cytotoxicity of PG-TXL and to the enhancement of tumor radiation response. This treatment provided significant therapeutic gain, better than other taxanes thus far tested *in vivo*. Thus, PG-TXL has a high potential to improve clinical radiotherapy.

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